Inhibition of Heat Shock Protein 90 Machinery for the Treatment of Cancer: Progress in the Development of Alternative Strategies

By

Jessica A. Hall

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Dr. Brian S. J. Blagg
Chairperson

_____

Dr. Emily E. Scott

_____

Dr. Michael F. Rafferty

_____

Dr. Rick T. Dobrowsky

_____

Dr. Teruna J. Siahaan

Date Defended: May 13th, 2015

The Dissertation Committee for Jessica A. Hall

certifies that this is the approved version of the following dissertation:

Inhibition of Heat Shock Protein 90 Machinery for the Treatment of Cancer: Progress in the Development of Alternative Strategies

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Dr. Brian S. J. Blagg
Chairperson

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Abstract

Heat shock proteins (Hsps) are molecular chaperones that facilitate the conformational maturation of newly synthesized and unfolded cellular proteins (termed “clients”) to maintain protein homeostasis. Heat shock protein 90 (Hsp90) is a chaperone that folds client substrates, many of which drive signal transduction pathways associated with cellular proliferation and differentiation. Consequently, Hsp90 can facilitate oncogenic transformation and can sustain the proper functioning of signaling pathways that have been hijacked during cancer formation and progression. Hsp90 functions as a homodimer and facilitates client maturation via the Hsp90 chaperone cycle. During this cycle, Hsp90 forms a heteroprotein complex with additional proteins (e.g., co-chaperones, partner proteins, immunophilins, etc.) that assist in client folding at different stages of the cycle. However, if this cycle is disrupted and a client is unable to reach conformational maturity, the immature client is ubiquitinylated and degraded via the proteasome.

Given Hsp90’s role in cancer progression, Hsp90 inhibition has emerged as a viable strategy for the development of anticancer chemotherapeutics. Classic Hsp90 inhibitors compete with ATP at the Hsp90 N-terminus, of which ATP-binding and hydrolysis is crucial for client maturation. However, these N-terminal inhibitors lead to induction of the pro-survival heat shock response via activation of the transcription factor, heat shock factor-1 (HSF-1; resides at the Hsp90 N-terminus). HSF-1 activation ultimately increases the cellular concentration of Hsps, including Hsp90. Therefore, alternative strategies to inhibit Hsp90 function and/or client maturation that avoid HSF-1 activation (i. e. increased Hsp90 levels) have been pursued.

An alternative strategy to inhibit Hsp90-dependent client maturation is to target the Hsp90 C-terminus. The natural products novobiocin and (-)-epigallocatechin-3-gallate (EGCG)

were previously identified as Hsp90 C-terminal inhibitors and provide platforms for elucidation of structure-activity relationship studies for the Hsp90 C-terminus. These natural products led to development of analogs that exhibited potent anti-proliferative activity across several different cancer cell lines, many of which decreased the cellular levels of Hsp90 clients and did not affect Hsp levels.

Another strategy to prevent Hsp90 client maturation is to disrupt components of the heteroprotein complex, specifically the interactions between Hsp90 and its co-chaperones. The known F_1F_0 ATP synthase inhibitor, cruentaren A, was shown to disrupt interactions between the co-chaperone F_1F_0 ATP synthase and Hsp90 via F_1F_0 ATP synthase inhibition. Disruption of this interaction led to decreased client levels and no increase in Hsp levels. Other disruptors of Hsp90 client maturation include members of the cucurbitacin class of natural products, specifically cucurbitacin D and 3-epi-isocucurbitacin D. These cucurbitacins led to decreased client protein levels without HSF-1 activation. However, only cucurbitacin D disrupted interactions between Hsp90 and the co-chaperones, Cdc37 and p23, similar to the known heteroprotein complex disruptor, gedunin.

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Table of Contents

List of Sections:

Chapter III

Identification of Natural Products that Disrupt Hsp90-dependent Client Protein Maturation

Chapter IV

Continued Optimization of Novobiocin-derived Hsp90 C-Terminal Inhibitors

List of Figures:

Chapter I

Alternative Approaches to Hsp90 Modulation for the Treatment of Cancer

Chapter II

Cruentaren A Binds F1F0 ATP Synthase to Modulate the Hsp90 Protein Folding Machinery

Chapter III

Identification of Natural Products that Disrupt Hsp90-dependent Client Protein Maturation

Chapter IV

Continued Optimization of Novobiocin-derived Hsp90 C-Terminal Inhibitors

Appendix B

Appendix C

List of Schemes:

Chapter III

Identification of Natural Products that Disrupt Hsp90-dependent Client Protein Maturation

List of Tables:

Chapter I

Alternative Approaches to Hsp90 Modulation for the Treatment of Cancer

Chapter II

Cruentaren A Binds F1F0 ATP Synthase to Modulate the Hsp90 Protein Folding Machinery

Chapter III

Identification of Natural Products that Disrupt Hsp90-dependent Client Protein Maturation

Chapter IV

Continued Optimization of Novobiocin-derived Hsp90 C-Terminal Inhibitors

Appendix B

Appendix C

List of Appendices:

Appendix A

Appendix B

Appendix C

Chapter I

Alternative Approaches to Hsp90 Modulation for the Treatment of Cancer

I.1 Molecular Chaperones and Anticancer Chemotherapeutics

On average, mammalian cells express over 10,000 different proteins that are synthesized by ribosomes as linear polypeptide chains consisting of thousands of amino acids. In order for these linear amino acids chains to function properly, they must 'fold' into single, biologically active, three-dimensional structures.[1, 2] Proper folding of proteins into conformational states that promote normal cellular function is crucial for maintaining cellular homeostasis. Aberrant structures can lead to fibrillization and contribute to disease states associated with neurological decline. Therefore, protein quality control and the maintenance of proteome homeostasis are critical to cellular and overall health of an organism. Deficiencies in protein homeostasis have been shown to cause or progress numerous diseases, such as neurodegeneration and dementia, type 2 diabetes, peripheral amyloidosis, lysosomal storage disease, cystic fibrosis, cancer and cardiovascular disease.[3] A network of several hundred proteins, notably molecular chaperones and their regulators, assist in *de novo* folding or re-folding.[4]

Molecular chaperones are an evolutionarily conserved class of proteins that prevent aggregation and assist in the conformational maturation of other cellular proteins (referred to as client proteins). Heat shock proteins (Hsps) are a group of molecular chaperones that are ubiquitously expressed under non-stressed conditions and upregulated upon exposure to cellular stress, including elevated temperature. Hsp90 is the most abundant heat shock protein and represents 1-2% of total cellular proteins in unstressed cells.[5-7] There are four human isoforms of Hsp90; the cytosolic isoforms Hsp90α and β, Grp94 (localized to the endoplasmic reticulum) and TRAP1 (localized to the mitochondria). Hsp90 facilitates the conformational maturation of

Hsp90-dependent proteins via the Hsp90 chaperone cycle, in which the Hsp90 homodimer forms a large, multiprotein complex that relies upon co-chaperones, immunophilins, and partner proteins to fold nascent polypeptides, as well as the rematuration of denatured proteins.[8-10] The Hsp90 heteroprotein complex folds these substrates through a series of conformational transitions at the middle and N-terminal domains of Hsp90 that are facilitated by ATP hydrolysis at the N-terminus.[11, 12] Inhibition of the Hsp90 protein folding machinery results in client protein ubiquitinylation and subsequent degradation via the proteasome, which can ultimately result in cell death.[13-16] Many Hsp90-dependent client proteins (e.g. ErbB2, B-Raf, Akt, steroid hormone receptors, mutant p53, HIF-1, survivin, telomerase, etc.) are associated with the six hallmarks of cancer (Table 1).[17] Therefore, oncogenic client protein degradation via Hsp90 inhibition represents a promising approach toward anticancer drug development.[18-20]

Table 1 Hsp90-dependent clients and the hallmarks of cancer

Hallmark of Cancer	**Hsp90-Dependent Client**
Invasion and Metastasis	MMP2, Urokinase
Self-sustained Growth Signals	ERBB2, EGFR, KIT, RTKs, CRAF, BRAF
Insensitivity to Anti-growth Signals	CDK4, CDK6, Cyclin D
Evasion of Apoptosis	IGF-1R, AKT, survivin
Limitless Replication potential	*h*TERT
Sustained Angiogenesis	HIF, MET, SRC, VEGFR, additional RTKs

Originally, small molecule inhibitors of Hsp90 were designed to perturb the ATPase activity located at the N-terminus and include derivatives of geldanamycin, radiciol and

purine.[21-23] N-terminal Hsp90 inhibitors are effective at inhibiting Hsp90 function and lead to anti-proliferative activity through client protein degradation; however, Hsp90 N-terminal inhibition also leads to induction of the Heat Shock Response (HSR).[24-26] N-terminal inhibitors displace the Hsp90-bound transcription factor, Heat Shock Factor-1 (HSF-1).[27, 28] Upon displacement, HSF-1 trimerizes, translocates to the nucleus and binds the Heat Shock Element, which leads to transcriptional activation of the heat shock proteins, including Hsp90. The HSR is a pro-survival response to conditions that cause the denaturation of proteins. Hsp27, Hsp40, Hsp70, and Hsp90, amongst other Hsps, are overexpressed to refold denatured proteins. The consequence of inducing a pro-survival response concomitantly with inducing client protein degradation is typically, cytostatic activity. However, this induction also leads to dosing and scheduling problems in the clinic, as N-terminal inhibitors induce expression of the target they inhibit. Therefore, Hsp90 inhibitors with novel mechanisms of action are sought to take advantage of the dependence that client protein-driven cancers have upon Hsp90, without concomitant induction of the pro-survival, heat shock response.

Two alternative strategies for inhibiting the function of Hsp90 include disruption of the Hsp90 heteroprotein complex and disruption of the Hsp90 C-terminal dimerization domain. Disruption of the Hsp90 heteroprotein complex has emerged as an effective strategy to prevent client protein maturation without induction of the HSR.[29] More specifically, disruption of interactions between Hsp90 and co-chaperones, such as Cdc37, or direct inhibition of co-chaperones and immunophilins, such as p23, F_1F_0 ATP synthase and FKBP52, prevent the maturation of Hsp90 clients at concentrations that do not induce the HSR.[30-33]

In contrast, novobiocin was the first Hsp90 C-terminal inhibitor identified, and was found to weakly inhibit Hsp90 in SKBr3 cells at approximately 700 μM concentration.[34] As a result,

derivatives of the coumarin-containing natural product have been intensely sought and compounds manifesting improved activity identified. These C-terminal inhibitors prevent cancer cell proliferation at concentrations similar to N-terminal inhibitors and induce degradation of Hsp90-dependent client proteins without induction of the HSR.[35, 36] In addition to novobiocin and novobiocin analogs, epilgallocatechi-3-gallate (EGCG), silybin and cisplatin have been reported to bind the Hsp90 C-terminus.[37]

Within this chapter, recent progress in Hsp90 structure elucidation and dynamic aspects of the Hsp90 chaperone cycle will be discussed. Alternative strategies for Hsp90 inhibition that include direct and indirect targets of Hsp90, as well as the current state of traditional small molecule Hsp90 inhibitors will be summarized. Lastly, post-translational modifications to Hsp90 and partner proteins will be described as well as the effect of these modifications on client protein maturation, stability and activity in normal versus transformed cells.

I.2 Hsp90 Chaperone Cycle

Hsp90 primarily exists as a homodimer composed of two Hsp90 monomers that each contains three domains: an N-terminal domain with an ATP-binding pocket, a C-terminal domain responsible for dimerization, and a middle domain that connects the N- and C-termini through a flexible, highly charged linker. The cycle in which Hsp90 folds client proteins is highly complex (illustrated in Figure 1). Various co-chaperones are needed for regulation of the protein folding cycle and assist in the many conformational adjustments needed to obtain the catalytically active state of Hsp90.[38] Many of the co-chaperones that associate with Hsp90 at the beginning of the chaperone cycle bind the EEVD motif located at the Hsp90 C-termini. The co-chaperone Hsp90-Hsp70 organizing protein (HOP) binds Hsp90 to stabilize the open conformation and is responsible for delivering substrates from Hsp70 to Hsp90. A brief overview

of co-chaperone binding to Hsp90, and their effects are highlighted in Table 2.[39] HOP interacts directly with the EEVD motif through its tetratricopeptide repeat (TPR) domain.

Table 2 Hsp90 co-chaperones and effects on Hsp90 conformation and ATPase activity

Co-chaperone	Interaction site in co-chaperone	Interaction site in Hsp90	Effect on Hsp90 conformation	Effect on Hsp90 ATPase activity
Hop/Sti1	TPR2A, TPR2B	N, M, C	Stabilizes open state	Inhibition
Cdc37	M, C	N	Stabilizes open state	Inhibition
Sgt1	CS domain	N	Not determined	No effect
Tah1	TPR	C	Not determined	Weak inhibition by Tah1-Pih1 complex
Aha1	N, C	N, M	Stabilizes closed state	Acceleration
Ppt/PP5	TPR1, TPR2, TPR3	C	Not determined	No effect
Cpr6, Cpr7/Cyp40	TPR	C	Not determined	Weak acceleration
FKBP51/FKBP52	TPR	C	Not determined	No effect
p23/Sba1	N	N	Stabilizes closed state	Inhibition

TPR tetratricopeptide repeat, CS = CHORD (cysteine and histidine-rich domain)-containing protein and Sgt1 domain; N = N-terminal domain, M = middle domain, C = C-terminal domain

Once HOP is bound, Hsp70 and the client protein stabilize the open conformation of Hsp90. Various immunophilins (those which possess prolyl isomerase activity), co-chaperones, and other partner proteins associate with this Hsp90 conformation and form a heteroprotein complex. An asymmetric complex is formed when immunophilin co-chaperones, like peptidyl-prolyl isomerases (e. g. FKBP51, FKBP52, etc.), bind Hsp90.[39] This asymmetric structure is referred to as the 'late complex' and promotes chaperone cycle activity via stabilization of the open conformation.[40] After late complex formation, Hsp90 ATPase activator, Aha1, becomes part of the heteroprotein complex and facilitates the dissociation of other co-chaperones, such as

HOP and Hsp70.[40] Buchner and coworkers demonstrated that Aha1, the most effective activator of ATPase activity, is required for HOP release, as opposed to p23, which was previously reported.[40] These investigators found that Aha1 and the co-chaperone Cpr6 synergistically bind Hsp90 to stabilize the Hsp90 closed conformation.

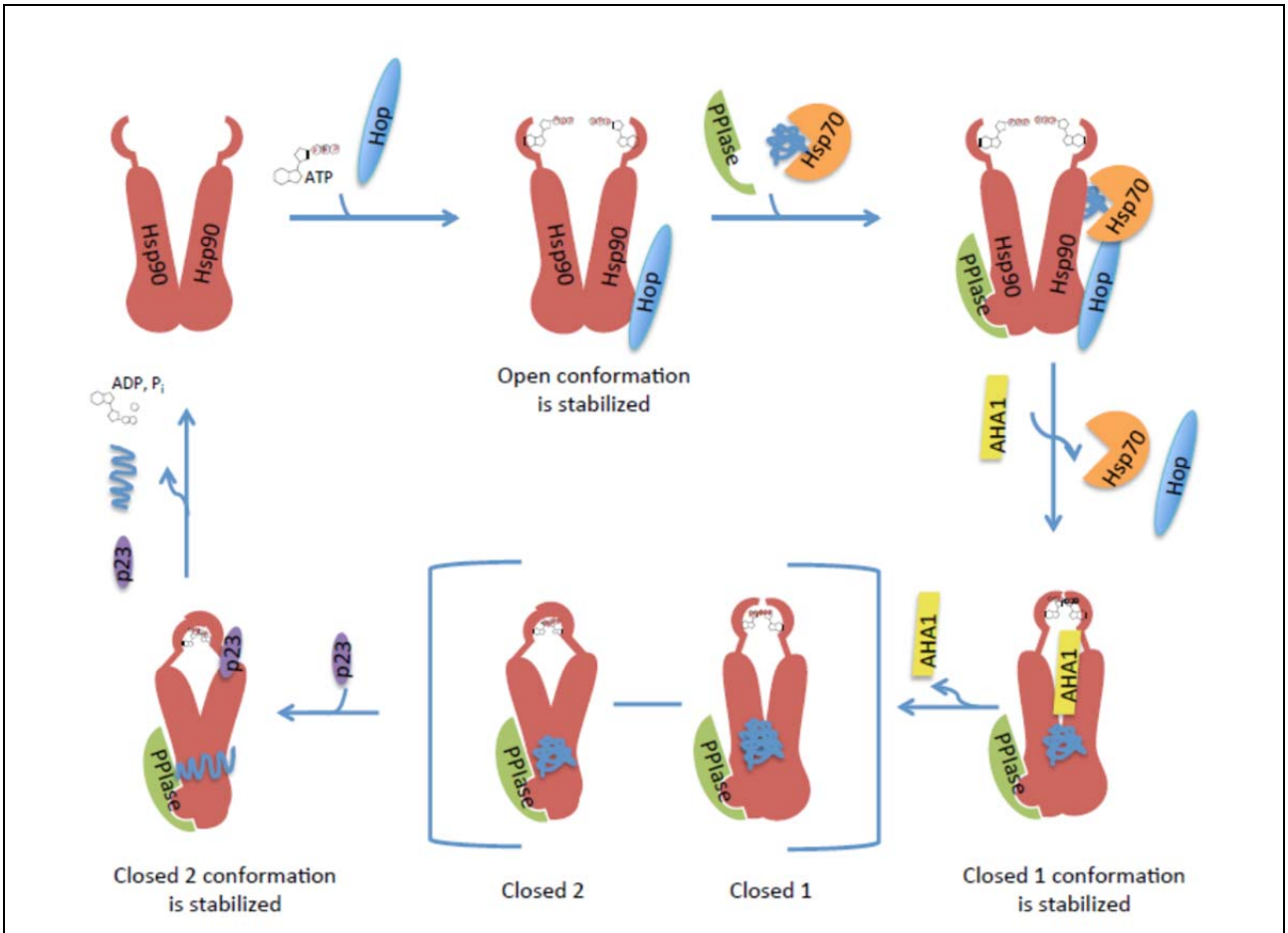

Figure 1 Hsp90 catalytic chaperone cycle, highlighting the two closed conformations. The first closed conformation is stabilized by Aha1. p23 then displaces Aha1, to form the second closed conformation.[38, 41]

Hydrolysis of ATP by Hsp90 is required for release of the mature protein substrate in addition to correct protein folding. Hsp90 must adopt a closed conformation in order to achieve ATP hydrolysis.[38] To form this closed state, Aha1 association occurs first, followed by ATP binding. Structural reorganization then facilitates closure of the N-terminal ATP lid. Lid closure stabilizes the catalytic loop of Hsp90, which is required for ATP hydrolysis. Furthermore, the closed N-terminal lid creates contacts between specific residues of the catalytic loop and ATP, as well as contacts with the adjacent Hsp90 monomer. This ultimately results in further stabilization of the Hsp90 complex. Conformation changes required for N-terminal lid closure represent the rate-limiting step in the protein folding cycle, as opposed to ATP hydrolysis.[38]

Aha1 not only binds in a synergistic manner with Cpr6 to Hsp90, Aha1 association accelerates the conformational changes required to attain the closed state. Interestingly, the heteroprotein complex conformation formed upon Aha1 binding is different from the state wherein ATP hydrolysis occurs, (refer to Figure 1).[40] In the Aha1-bound closed state, ATP can exchange readily. However, upon p23 displacement of Aha1, a second closed conformation is formed, in which ATP hydrolysis occurs.[40] The resulting complex dissociates and the folded substrate is released to promote repetition of the catalytic cycle.

p23 is a well-established regulator of Hsp90 ATPase activity and has been shown to stabilize the ATP-bound closed conformation of Hsp90 and inhibit client release.[42, 43] McLaughlin et. al demonstrated that p23 interacts with Hsp90 in the presence and absence of ATP; however, affinity for p23-Hsp90 complex formation increased when ATP is present. Mass spectral analysis determined that one molecule of p23 bound to each Hsp90 monomer and p23 interacted with truncated Hsp90 fragments that lack the C-terminus. These data indicate that Hsp90 dimerization is not required for p23-Hsp90 association. Collectively, these data suggest

that p23 locks Hsp90 subunits during the ATP-bound state, which exhibits a high affinity for protein substrates.

Another co-chaperone, carboxyl terminus of Hsp70-interacting protein (CHIP), regulates the release of properly folded client proteins, or targets them for degradation.[44, 45] CHIP contains a ubiquitin ligase domain, and thus provides a direct link to protein degradation via the proteasome.[46] CHIP interacts with Hsp90 or Hsp70 through its TPR domain, and when bound to Hsp90, CHIP can displace HOP and p23. In the presence of Hsp90 inhibitors, CHIP mediates ubiquitinylation of substrates and ultimately directs them for degradation via the proteasome.[47]

Other co-chaperones have less defined roles in the inhibition or promotion of Hsp90 ATPase activity; however, proper function of these co-chaperones and direct interaction with Hsp90 appear important for client protein maturation. Small glutamine-rich tetratricopeptide repeat-containing protein α (SGTA) is linked to several cellular processes including cell division, mitosis, cell cycle checkpoint activation, and viral infection. SGTA interacts directly with Hsp70 and may interact with Hsp90 via its TPR domain to facilitate maturation of the growth hormone and androgen receptors.[48] It has also been proposed that SGTA participates in the sequestration of client proteins in an inactive state.

F_1F_0 ATP synthase was proposed to possess co-chaperone function by interacting with Hsp90 in several different cancer cell lines.[33, 49] Furthermore, inhibition of F_1F_0 ATP synthase directly affected client protein maturation. F_1F_0 ATP synthase is a macromolecular machine that produces the majority of cellular ATP and is localized to the mitochondria and, in some cancers, the cell surface.[50-53] F_1F_0 ATP synthase was shown to directly interact with Hsp90, specifically the Hsp90α isoform.[54] A selective inhibitor of F_1F_0 ATP synthase, as well as general inhibitors of

ATP synthases, have been shown to disrupt interactions between F_1F_0 ATP synthase and Hsp90α, and ultimately result in client protein degradation.[54, 55]

I.3 Client-specific Hsp90 Co-chaperones

While Hsp90-co-chaperone interactions continually change throughout the chaperone cycle to either inhibit or promote ATP hydrolysis, co-chaperones also exhibit client specificity during the chaperone cycle and promote the maturation of client protein classes.[56] The mechanism by which co-chaperones assist in the delivery of clients to Hsp90 may occur via interactions with specific clients or by interactions with Hsp90 that prime the chaperone machinery for selective loading of protein substrates.

The co-chaperone activity manifested by cell division cycle 37 homolog, or Cdc37, is required for the maturation of all Hsp90-dependent kinases.[30, 57] This co-chaperone inhibits ATPase activity, but does not affect accessibility to the ATP binding site.[58-60] Sgt1 (suppressor of G2 allele of skp1) is required for activation of nucleotide-binding leucine-rich repeat receptors (NLRs).[61] The co-chaperones Tah1 (TRP-containing protein associated with Hsp90) and Pih1 (protein interacting with Hsp90) are associated with chromatin remodeling and temporarily halt the Hsp90 ATPase cycle to prepare Hsp90 for the maturation of small nuclear ribonucleoproteins.[62]

Other co-chaperones that exhibit client protein specificity are the peptidyl-prolyl isomerases (PPIases), and include FK506 binding protein (FKBP) 51, FKBP52, and cyclophylin (Cyp) 40.[63, 64] These remodeling PPIases are commonly utilized in Hsp90-steroid hormone receptor complexes. Interestingly, these PPIases are selective for specific hormone receptor clients. FKBP51 in concert with protein phosphatase 5 (Pp5) is present during the maturation of

glucocorticoid receptor complexes; however, isolated FKBP51 is found associated with progesterone receptor, whereas Cyp40 associates with the estrogen receptor.

While it remains unknown as to how co-chaperones selectively interact with clients, co-chaperones involved in the maturation of certain clients, or substrate classes, provide an opportunity to selectively prevent the maturation of these client(s) by targeting interactions between Hsp90 and the co-chaperone.

I.4 Disrupting Hsp90-co-chaperone Interactions

Disruption of interactions between Hsp90 and co-chaperones has been a successful strategy for selective and non-selective degradation of client protein substrates. Small molecules can disrupt these interactions and manifest properties unlike N-terminal Hsp90 inhibitors. Celastrol and gedunin (Figure 2) were originally suspected to disrupt interactions between Hsp90 and Cdc37 and induce degradation of kinase client proteins.[65-67] The mechanism by which these compounds affect the Hsp90 chaperone cycle has since been explained. Celastrol is a natural product that exerts potent anti-cancer activity via multiple mechanisms of action. One of these mechanisms is disruption of Hsp90-Cdc37 interactions. The natural product gedunin was shown to disrupt Hsp90-co-chaperone interactions through binding p23.

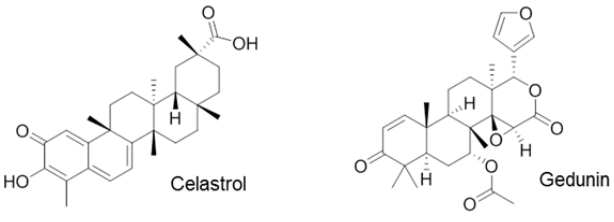

Figure 2 Structures of celastrol and gedunin.

NMR studies by Sreeramulu, et. al. demonstrated that celastrol binds and covalently modifies cysteine residues located at the N-terminus of Cdc37 via Michael adduct formation and therefore, does not directly bind Hsp90.[68] In addition, Chadli, et. al. report that celastrol inhibits the Hsp90 chaperone cycle by destabilizing p23, which results in the degradation of steroid hormone receptors.[69] Therefore, celastrol appears to exhibit a multi-faceted mechanism for inhibition of the Hsp90 protein folding cycle. In addition, it was reported that gedunin binds the co-chaperone, p23, and inhibits p23 chaperone activity while blocking interactions between p23 and Hsp90.[32] Gedunin inactivated p23 via caspase 7 activation, which led to cleavage of the p23 C-terminus, resulting in apoptosis. Interestingly, treatment with gedunin did not increase Hsp27 levels and only slightly elevated Hsp70 levels were observed, suggesting this approach may not induce the HSR.

Hsp90 requires ATP for chaperone activity and inhibition of ATP synthase with pan ATP synthase inhibitors such as oligomycin A, 2-deoxy-D-glucose, antimycin A and efrapeptins prevent Hsp90-dependent client maturation, which destabilizes the client-Hsp90 complex, leading to client degradation via the proteosome (Figure 3).[49, 54, 55] ATP synthase inhibitors do not induce the HSR, as oligomycin A and efrapeptins manifested little to no increase in Hsp90, Hsp70 and Hsp27 levels. Furthermore, inhibition of the singular ATP synthase, F_1F_0 ATP synthase, with the selective inhibitor, cruentaren A, retained this activity and resulted in client protein degradation through destabilization of F_1F_0 ATP synthase-Hsp90α interactions (Figure 3).[54, 70, 71] Hsp90 and p23 in complex with hTERT, the catalytic subunit of telomerase, is required for the nuclear localization of telomerase, a protein whose unregulated function contributes to unlimited replicative potential. The promiscuous natural product, curcumin, induced cytoplasmic accumulation and degradation of hTERT through disruption of p23-hTERT

interactions as one of its many mechanisms of action (Figure 3).[72] Curcumin decreased association between p23 and hTERT, but did not prevent hTERT from interacting with Hsp90. In contrast, the N-terminal Hsp90 inhibitor, geldanamycin, disrupted both the Hsp90-hTERT and p23-hTERT complexes.

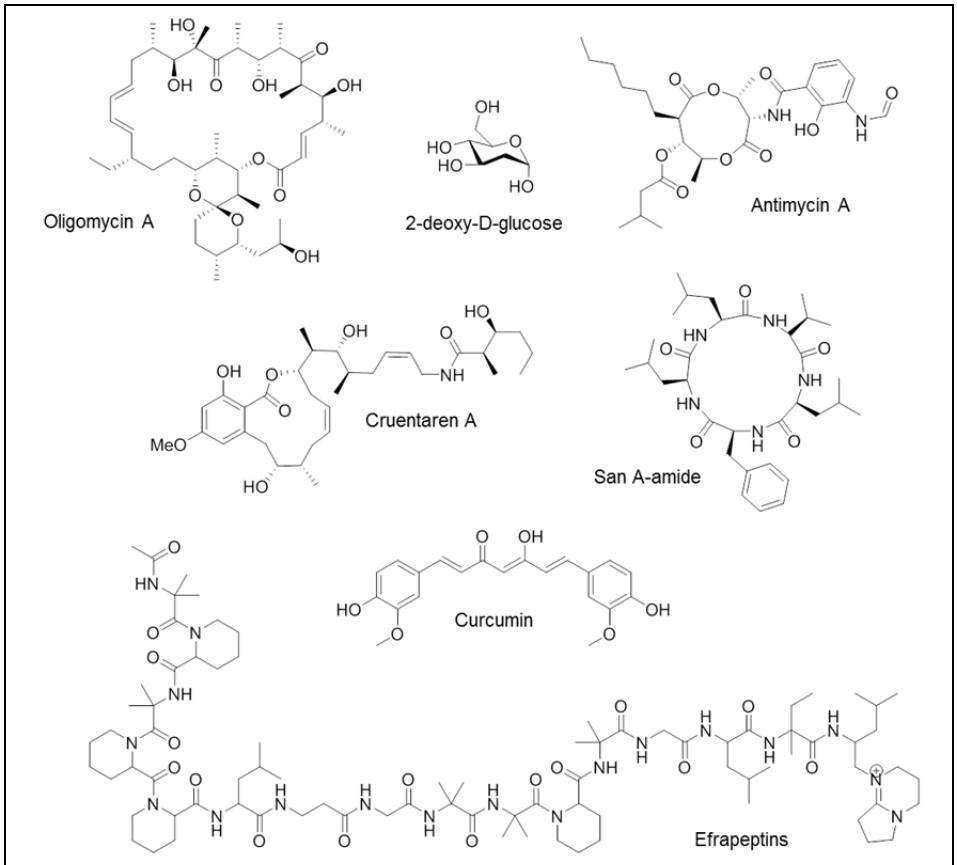

Figure 3 Structures of allosteric modulators of the Hsp90 chaperone cycle.

San A-amide, a derivative of the natural product sansalvamide A, induces apoptosis in various cancer cell lines, including pancreatic, colon, breast and prostate (Figure 3).[73, 74] Vasko et. al. reported that San A-amide-mediated apoptosis in the HCT-116 colon cancer cell line occurred through displacement of inositol hexakisphosphate kinase-2 (IP6K2) and FKBP52 from

the Hsp90 C-terminus.[75] San A-amide binds the N-middle domain of Hsp90 and appears to disrupt the structural equilibrium of this region, which appears to alter the substrate binding site. In contrast, San A-amide has no effect on the N-terminal client, Her2. This mechanism of action is unique to San A-amide, as the Hsp90 N-terminal inhibitor 17-allylaminogeldanamycin (17-AAG) does not affect IP6K2 and FKBP52 binding, but does affect Her2 maturation. Furthermore, San A-amide exhibits no effect on Hsp90 ATPase activity, which supports its role as an allosteric modulator and its potential to selectively disrupt Hsp90 C-terminal binding interactions.

I.5 N-Terminal Hsp90 Inhibitors

Hsp90 inhibitors that bind the N-terminus compete with ATP and perturb ATPase activity, thereby disrupting client protein maturation. ATP adopts a specific conformation when bound to Hsp90, due to the presence of a Bergerat fold in the protein structure, which is characteristic of the GHKL (Gyrase, Hsp90, Histidine, Kinase, MutL) subgroup within the ATPase/kinase superfamily.[76, 77] Compounds that bind the N-terminal ATP-binding pocket adopt a bent conformation and afford selectivity for Hsp90 over other ATP-binding proteins. All Hsp90 inhibitors under clinical investigation are N-terminal inhibitors and include derivatives of geldanamyicn (GDA), radicicol (RDC), and purine (Figure 4). N-terminal inhibitors have been studied in great detail and comprehensive reviews are present throughout the Hsp90 literature.[78-82]

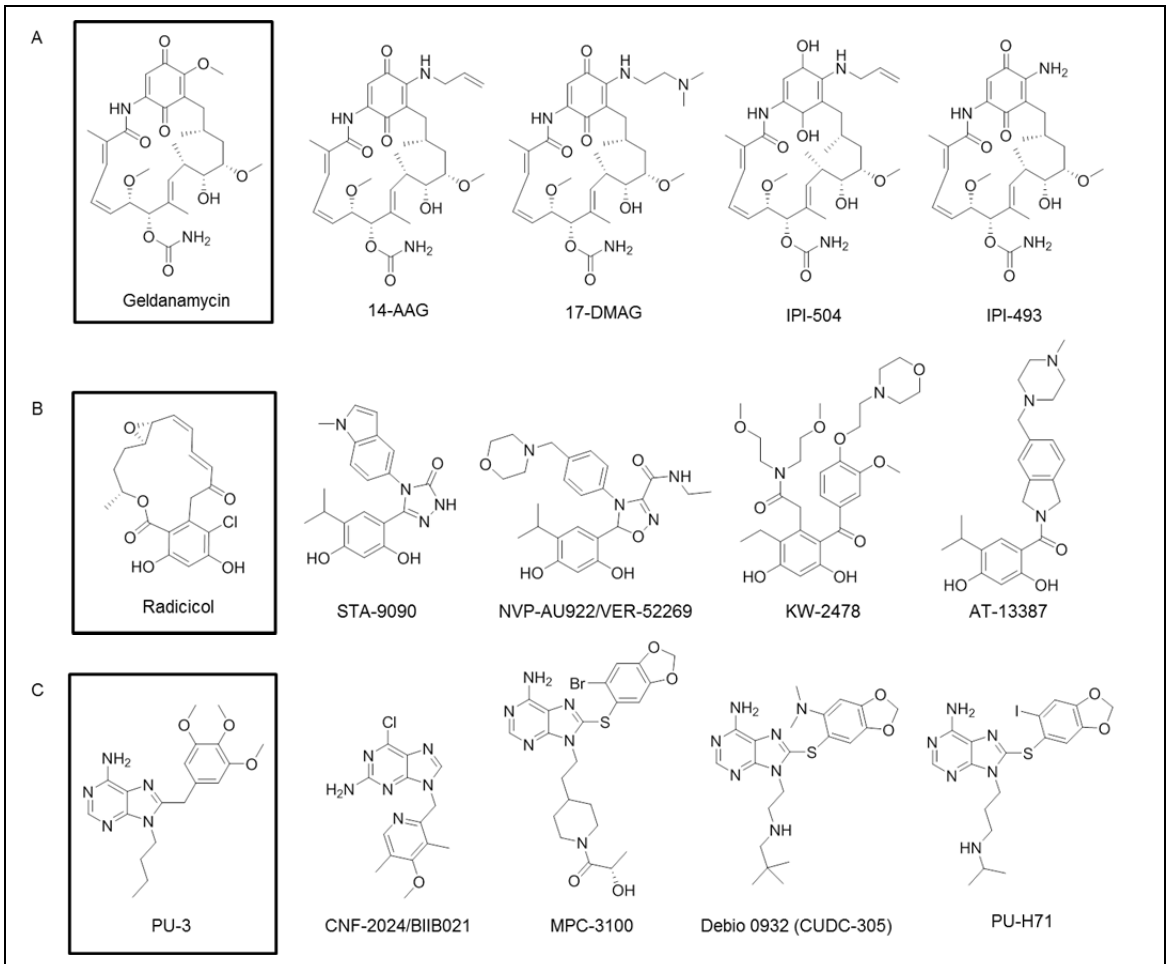

Figure 4 Structures of geldanamycin and derivatives (A), radicicol and derivatives (B), as well as PU-3 and purine-containing analogs (C).

GDA belongs to the benzoquinone class of ansamycins and was originally pursued as an antibiotic until it was shown to reverse v-src oncogenic transformation by destabilization of the Hsp90-src complex.[83] Although GDA is a potent anticancer agent, it did not undergo clinical evaluation due to its poor drug-like properties. However, several derivatives of GDA have entered clinical investigation and include 17-allyl-17-demethoxygeldanamycin (17-AAG), 17-desmethoxy-17-N,N-dimethylaminoethylaminogeldanamycin (17-DMAG), 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), and 17-desmethoxy-17-

aminogeldanamycin (IPI-493) (Figure 4A). RDC is a macrocyclic lactone that contains a resorcinol moiety as well as an allylic epoxide and an α,β,γ,δ-unsaturated ketone. Although RDC is unstable in serum, the resorcinol moiety of RDC has been utilized to develop several compounds that are under clinical investigation, such as STA-9090, NVP-AU922/VER-52269, KW-2478, and AT-13387 (Figure 4B). The crystal structure of ADP bound to Hsp90 provided an opportunity to design inhibitors that take advantage of the unique Bergerat fold. Purine-containing compounds, such as PU-3, CNF 2024/BIIB021, MPC-3100, Debio 0932 (CUDC-305), and PU-H71 are also undergoing clinical evaluation (Figure 4C).

Despite the effectiveness of N-terminal inhibitors as anticancer agents, several concerns have emerged as a consequence of N-terminal inhibition. Induction of the HSR has resulted in dosing and scheduling problems during clinical trials and spurred the development of Hsp90 inhibitors that are devoid of this response, such as C-terminal inhibitors and allosteric modulators of the chaperone cycle. In addition, gastrointestinal distress, hepato- and ocular toxicities have been observed during pre-clinical and clinical trials.[84] This may be the result of off-target affects, metabolic instability and/or pan inhibition of all four Hsp90 isoforms. Recently, it was shown that the hERG channel depends upon the Hsp90α isoform for its maturation.[85] Hsp90 inhibitors have been shown to disrupt proper trafficking and function of hERG.[86] Therefore, cardiotoxicity has also been a concern during the development of Hsp90 inhibitors. All Hsp90 N-terminal inhibitors in the clinic exhibit pan Hsp90 inhibitory activity and target all four isoforms. It appears that other clients (oncogenic or not) may also exhibit a preference for individual Hsp90 isoforms.[85, 87, 88] Therefore, deciphering the role played by each isoform will be important for the development of future Hsp90 inhibitors.

Despite the high sequence similarity and identity possessed among the N-terminal regions of each Hsp90 isoform, inhibitors have recently been developed that manifest selective inhibition. An inhibitor derived from the ansamycin scaffold has been developed that targets Hsp90α and β, but not Grp94 (Figure 5A).[89] Likewise, resorcinol- and purine-containing inhibitors have also been reported to selectively inhibit Grp94 based on subtle differences within the N-terminus of Grp94 (Figure 5B and C).[87, 88]

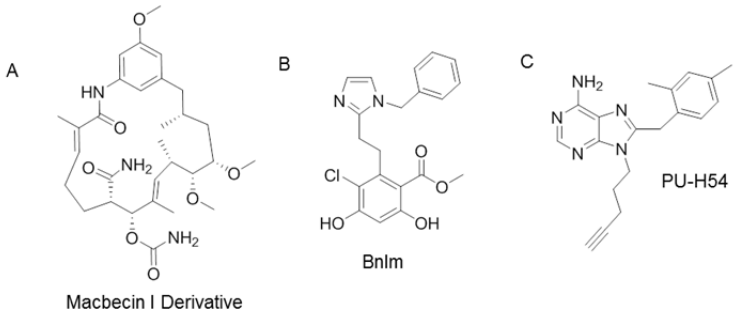

Figure 5 Structures of Hsp90α/β inhibitor (A), resorcinol-containing Grp94 inhibitor (B), and purine-containing Grp94 inhibitor (C).

I.6 Post-translational Modifications of the Hsp90 Chaperone Cycle

The Hsp90 chaperone cycle can be fine-tuned to quickly adapt to changes in the intracellular environment through post-translational modifications (PTMs). Several comprehensive reviews describe these covalent modifications, which include phosphorylation, acetylation, S-nitrosylation, oxidation, ubiquitinylation, and SUMOylation. These PTMs can affect ATPase activity, stabilization of Hsp90-client interactions, co-chaperone interactions, and in some cases, target client proteins for maturation or degradation.[90-92] A summary of some Hsp90 PTMs and original references are provided in Table 2. PTMs of the Hsp90 chaperone cycle can enhance or diminish the effects of small molecule Hsp90 inhibitors, such as GDA. Targeting these PTMs can provide an opportunity to enhance the efficacy of Hsp90 inhibitors

and gain further insight into the mechanism by which PTMs regulate the Hsp90 protein folding machinery.

Co-chaperones Cdc37, Sgt1, p23, and FKBP52 are susceptible to phosphorylation by casein kinase 2 (CK2).[93-98] CK2 phosphorylates Ser-13 of Cdc37. This modification is required for Cdc37's chaperoning of numerous kinase clients as well as for the binding of Cdc37 to Hsp90. Dephosphorylation of Cdc37 at Ser-13 by the phosphatase, PP5/Ppt1, negatively affects the chaperone cycle and prevents maturation of these clients. Phosphorylation of Sgt-1 at Ser-361 negatively impacts Sgt-1 dimerization and prevents kinetochore assembly, a process required for chromosome segregation during eukaryotic cell division. CK2-mediated phosphorylation of p23 at Ser-113 and -118 was shown to be important for prostaglandin synthase activity and required for formation of the Hsp90-p23-CK2 complex. CK2 also phosphorylated thr-143 of FKBP52. While this modification does not affect FKBP52 from binding FK506, phosphorylated FKBP52 does not bind Hsp90.

Several kinases are capable of phosphorylating Hsp90 and have varying effects on the chaperone cycle. Double-stranded DNA protein kinase (DNA-PK) phosphorylates Thr-5 and -7 of the Hsp90α N-terminal domain and may play a role in DNA damage. Client kinases also phosphorylate Hsp90 and may regulate their own chaperoning via a feed-back mechanism. B-Raf was shown to phosphorylate Ser-263 of Hsp90 in melanoma and Akt to phosphorylate Hsp90α and Hsp90β, Grp78 and Grp94, Hsp70, and protein disulfide isomerase (PDI). c-Src phosphorylates Hsp90β at Tyr-301 in response to vascular endothelial growth factor receptor-2 (VEGFR-2) activation and increases association between Hsp90β and eNOS. Protein kinase A (PKA) phosphorylates Thr-90 of Hsp90α and reduces association with eNOS, which results in both diminished eNOS activity and nitric oxide production. Phosphorylation of Thr-90 of

Hsp90α via PKA was also shown to mediate cellular secretion of Hsp90α and may play a role in wound healing and/or metastasis. Swe1[Wee1] kinase phosphorylates Tyr-38 of the N-terminus of Hsp90α when Hsp90 is in the "open conformation" and positively affects the ability of Hsp90 to chaperone a select group of client kinases (ErbB2, Raf-1, and Cdk4). However, phosphorylation of this residue also negatively affects GDA binding to the Hsp90 N-terminus.

CK2 phosphorylation of Ser-231 and-263 in the charged linker of Hsp90α, as well as the equivalent resides in Hsp90β (Ser-226 and -255) occurs in normal cells, but not leukemic cells. Several leukemogenic kinases, such as Bcr-abl, FLT3/D835Y, and Tel-PDGFRβ, suppressed phosphorylation of Hsp90β at these residues, which resulted in stable interactions between Hsp90β and apoptotic peptidase activating factor-1 (Apaf-1). Ultimately, this activity led to inhibition of apoptosome function and may contribute to resistance in leukemia. CK2 phosphorylation at these sites also disrupts the Hsp90-aryl hydrocarbon complex and destabilizes the aryl hydrocarbon receptor protein. CK2 was also shown to phosphorylate Thr-36 of Hsp90α. Mutation of this residue to a phospho-mimetic, aspartate, resulted in decreased chaperoning of several clients and reduced interactions between Hsp90α with Aha1.

Acetylation/deacetylation of Hsp90 has been investigated using HDAC inhibitors.[99-101] Acetylation of Hsp90 by p300 resulted in diminished interactions between Hsp90 and clients and ultimately, resulted in client instability and degradation. Mutation of lysine to acetylated lysine was shown to decrease interactions between Hsp90 and several clients/co-chaperones as well as to reduce ATP-binding. Deacetylation of Hsp90 has been shown to occur via several HDACs including HDAC6, HDAC1 and HDAC10, which correlate with the stabilization of Hsp90-client interactions and increased chaperone activity.[102-105]

Other PTMs include S-nitrosylation of Hsp90α at Cys-597 via nitric oxide, oxidation of Cys-572 by the oxidized lipid, 4-hydroxy-2-nonenal (4-HNE; is formed during cellular oxidative stress), ubiquitinylation, and more recently, SUMOylation. S-nitrosylation, oxidation, and ubiquitinylation decrease chaperone function by perturbing ATPase activity or destabilizing Hsp90-client interactions, which results in client degradation. Alternatively, SUMOylation of the conserved lysine reside, Lys-191, initiated recruitment of the ATPase activating co-chaperone, Aha1. In addition, SUMOylation facilitated binding of several Hsp90 N-terminal inhibitors and sensitized yeast and mammalian Hsp90 to inhibition.

Table 3 Post-translational modifications of human Hsp90			
Modification	**Catalytic Protein or Moiety**	**Amino Acid Residue**	**Effect on Hsp90 Chaperone Activity**
Phosphorylation/ dephosphorylation	DNA-PK	Thr-5 and -7 phosphorylation[106]	Unknown
	B-Raf	Ser-263 phosphorylation of Hsp90α[107]	Unknown
	Akt	Thr and Ser phosphorylation of Hsp90α, Hsp90β, and Grp94[108]	Unknown
	c-Src	Try-301 phosphorylation of Hsp90β[109]	Increased eNOS activity and NO production.
	PKA	Phosphorylation of Ser-452 of Hsp90β and Thr-90 of Hsp90α[110, 111]	Phosphorylation of Hsp90α decreased eNOS activity and NO production. Also stimulates cellular secretion of Hsp90α.
	CK2	Phosphorylation of Ser-231 and -263 of Hsp90α and Ser-226 and -255 of Hsp90β in untransformed cells but not leukemic cells[112, 113]	Phosphorylation of Hsp90α caused dissociation of Hsp90 and the aryl hydrocarbon receptor.
	Swe1[Wee1] Kinase	Tyr-38 phosphorylation of Hsp90α[114]	Increased chaperoning activity of select clients. Decreased GDA binding to Hsp90.
S-nitrosylation	NO	Cys-597 nitrosylation[115, 116]	Inhibited ATPase activity
Oxidation	4-HNE	Cys-572 oxidation[117]	Decreased chaperone activity
Ubiquitinylation	Ubiquitin pathway	Lys ubiquitinylation[118, 119]	Destabilized Hsp90 client interactions and resulted in client degradation.
SUMOylation	SUMOylation pathway	Lys-191 SUMOylation[90]	Initiated recruitment of Aha1 and facilitated binding of N-terminal inhibitors.

Tumor dependency on pathways regulated by Hsp90 provides clear rationale for the development of Hsp90 inhibitors. Hsp90 inhibition offers a multifaceted treatment strategy, which is in contrast to current cancer therapies that target a single signaling pathway. Despite the

potential of Hsp90 as a drug target, no Hsp90 inhibitor has been FDA approved. Alternative methods to target Hsp90 include inhibition of the C-terminus or allosteric modulation of the chaperone machinery.

Recent advances toward elucidation of the Hsp90 structure and intricacies of the chaperone cycle have provided details on the conformational changes required for heteroprotein complex formation during various stages of the chaperone cycle. PTMs of proteins involved in the chaperone cycle have shed light on how quickly Hsp90 adapts to changes in the cellular environment. Methods alternative to N-terminal inhibition for modulation of Hsp90 may provide an opportunity to select for oncogenic client degradation, while avoiding some of the negative consequences of N-terminal inhibition. The Hsp90 chaperone cycle and inhibitor development have become a diverse and dynamic field of study. As details of the chaperone cycle emerge, more information regarding the regulation of chaperone machinery in normal versus transformed cells will be discovered. Such studies are expected to lead to the development of tailored strategies for modulation of the Hsp90 chaperone machinery for the treatment of cancer as well as other diseases.

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Chapter II

Cruentaren A Binds F_1F_0 ATP Synthase to Modulate the Hsp90 Protein Folding Machinery

II.1 Hsp90 and the co-chaperone F_1F_0 ATP synthase

As previously mentioned, heat shock proteins (Hsps) are a subset of molecular chaperones that are upregulated upon exposure to cell stress, including high temperature. Heat shock proteins are also ubiquitously expressed under non-stressed conditions and play vital roles in *de novo* protein synthesis by folding nascent polypeptides, translocating proteins across membranes, and mediating protein turnover.[1-3] They also serve regulatory functions that include the post-translational regulation of signaling molecules, the activation of transcription factors, and the degradation of proteins via the ubiquitin-proteasome pathway.[1-3]

Hsp90 is the most abundant heat shock protein and represents approximately 1-2% of total cellular protein in unstressed cells. Four isoforms of human Hsp90 exist and include the cytosolic proteins, Hsp90α (inducible) and Hsp90β (constitutively expressed), the endoplasmic reticulum-associated glucose-related protein 94 (Grp94), and the mitochondrial-associated TNF receptor-associated protein 1 (TRAP1). Hsp90-dependent client proteins play key roles in cellular growth, survival and development. The list of Hsp90 clients extends beyond 200 reported proteins, many of which contribute to the six hallmarks of cancer.[4]

In contrast to current cancer therapeutics that target at a single signaling pathway, Hsp90 inhibition results in the simultaneous degradation of multiple oncogenic substrates and leads to a combinatorial attack on cancer. Overexpression of Hsp90 occurs in cancer cells and is

responsible for maintaining the homeostasis of the hostile environment caused by neoplastic transformation.[4-8] Upregulated Hsp90 levels provide an opportunity to selectively target cancer cells versus noncancerous cells, and provide the opportunity to reduce detrimental side effects.

Hsp90 facilitates the conformational maturation of client proteins via the Hsp90 chaperone cycle, in which the Hsp90 homodimer forms a larger, multiprotein complex that contains other co-chaperones, immunophilins, and partner proteins that together are responsible for folding Hsp90-dependent substrates. The Hsp90 heteroprotein complex folds its client proteins through a number of conformational transitions that are facilitated by ATP hydrolysis within the N-terminus of the protein.[9] The Hsp90 heteroprotein complex is predominant in cancer cells, whereas the Hsp90 homodimer is abundant in non-transformed cells.[8, 10] In addition, the Hsp90 heteroprotein complex exhibits approximately 200-fold increased affinity for ATP as compared to the Hsp90 homodimer.[11] Such attributes have led to the development of small molecule inhibitors of the N-terminal ATP-binding pocket, and include derivatives of geldanamycin, radicicol and purine. Although N-terminal inhibitors are effective at inducing client protein degradation, N-terminal inhibition also leads to activation of the Hsp90-associated transcription factor, Heat Shock Factor-1 (HSF-1). HSF-1 activation induces the pro-survival heat shock response (HSR), in which the cellular concentration of heat shock proteins, such as Hsp90 and Hsp70, is dramatically increased, which can result in dosing and scheduling issues upon the administration of N-terminal inhibitors.[10, 12] Therefore, the development of small molecules that do not exhibit this mechanism of action are actively sought.

Alternative strategies for the inhibition of Hsp90 include the development of small molecules that target the Hsp90 C-terminus as well as the Hsp90 heteroprotein complex. C-

terminal inhibitors derived from the natural product novobiocin inhibit cancer cell proliferation and lead to client protein degradation at concentrations similar to N-terminal inhibitors, however they do not induce the HSR.[13-15] In contrast, small molecules that disrupt the Hsp90 heteroprotein complex, specifically interactions with co-chaperones, have been sought to disrupt maturation of select Hsp90 clients at concentrations that do not induce the HSR.[16-19] Papathanassiu, et. al. have reported that F_1F_0 ATP synthase interacts with Hsp90 to function as a co-chaperone that is important for the maturation of Hsp90 client proteins.[20] They showed that interactions between Hsp90 and F_1F_0 ATP synthase could be disrupted upon incubation with the polypeptide natural products, efrapeptins (Figure 6). Incubation with the efrapeptins resulted in the degradation of select Hsp90 clients after 48 hours and decreased cellular levels of Hsp70, Hsp90 and Hsp27. Efrapeptins represent a family of fungal peptides that exhibit potent anti-proliferative activity against several cancer cell lines by inhibiting the function of many cellular targets, including the 20S proteasome, several ATPases, and F_1F_0 ATP synthase.[21-23] Although efrapeptins induced Hsp90 client protein degradation at nanomolar concentrations, the complex peptide structure and non-specific inhibition hinders the development of these compounds. However, inhibition of F_1F_0 ATP synthase to ultimately disrupt interactions between Hsp90 and F_1F_0 ATP synthase represents a novel and under-investigated approach for disrupting Hsp90-dependent pathways without induction of the pro-survival heat shock response. Therefore, it was proposed that a selective inhibitor of F_1F_0 ATP synthase could inhibit the Hsp90 protein folding machinery via disruption of Hsp90-F_1F_0 ATP synthase interactions.

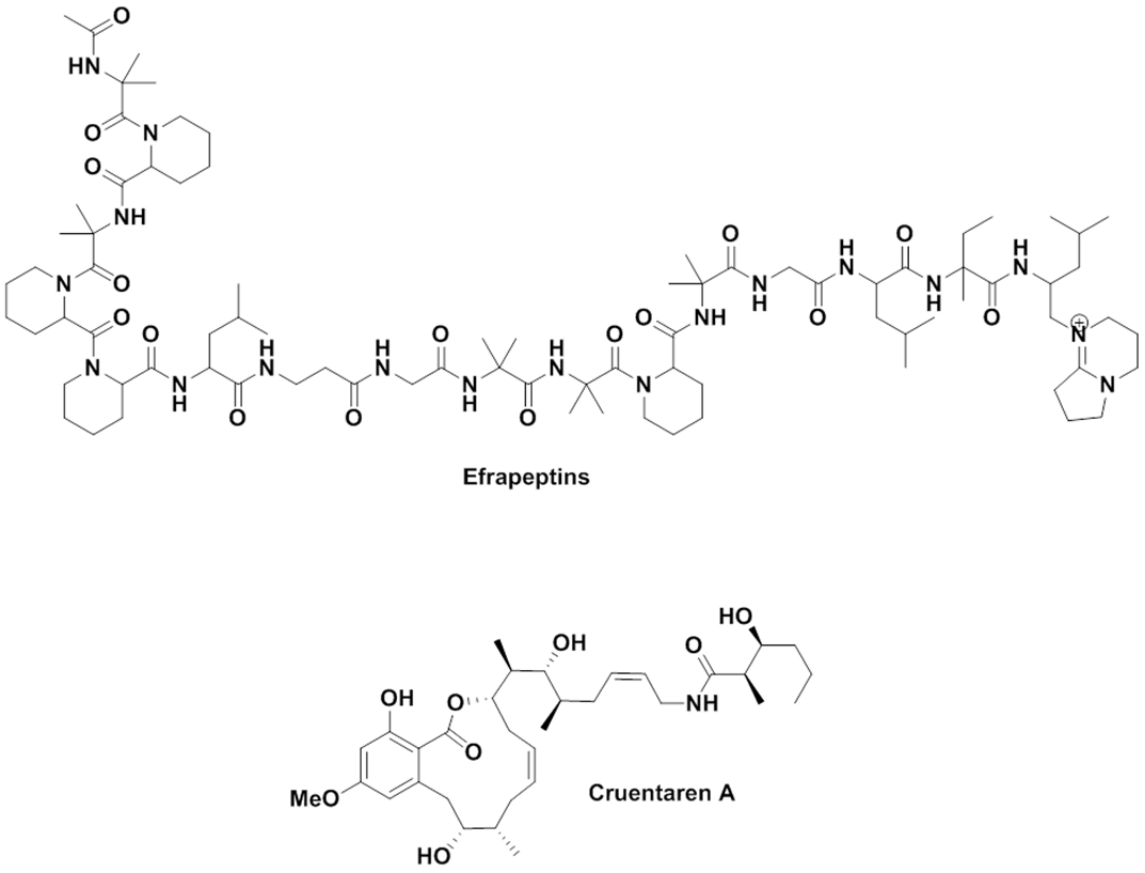

Figure 6 Structures of efrapeptins and cruentaren A.

The macrolide, cruentaren A, was isolated from the myxobacterium *Byssovorax cruenta* and shown to exhibit potent anti-proliferative activity against several cancer cell lines (Figure 6).[24, 25] Consistent with other cytotoxic natural products isolated from myxobacterium, cruentaren A was shown to be an inhibitor of oxidative phosphorylation and was found to be the only selective inhibitor of F_1F_0 ATP synthase identified. Studies revealed that cruentaren A selectively inhibits the F_1 domain of F_1F_0 ATP synthase and exhibits no inhibitory activity against other V- or P-ATPases. The inhibitory activity of cruentaren A also demonstrates

selectivity for eukaryotic F-ATPases, as it was completely inactive against a series of Gram-negative bacteria and did not inhibit the function of purified F_1 from *Escherichia coli*.

Consistent with this hypothesis, we found that incubation with nanomolar concentrations of cruentaren A resulted in Hsp90-dependent client protein degradation after 48 hours, and furthermore, did not induce the HSR after 24 or 48 hours of incubation.[26] As shown in these studies, cruentaren A does not bind directly to Hsp90 based on multiple assays. Instead, F_1F_0 ATP synthase was shown to interact directly with Hsp90 in MCF7 cell lysates, which could be disrupted upon 48 hour incubation with cruentaren A. In contrast, the N-terminal inhibitor, geldanamycin, and the C-terminal inhibitor, KU-174, did not affect interactions between F_1F_0 ATP synthase and Hsp90 after 24 and 48 hours of incubation, indicating that disruption Hsp90 chaperone function is specifically associated with F_1F_0 ATP synthase inhibition.

II.2 Cruentaren A is a potent inhibitor of cancer cell proliferation

Cruentaren A has been previously reported to demonstrate potent anti-proliferative activity against multiple cancer cell lines, including the human lung cancer cell line A549. Potent anti-proliferative activity against the A549 cell line as well as the estrogen receptor positive, human breast cancer cell line, MCF7 was observed; however, cruentaren A was relatively inactive against the normalized HEK293 and MRC5 human cell lines (Table 4 and Figure 7).

Table 4 Calculated EC_{50} values of cruentaren A against the cancer cell lines MCF7 and A549 and the normal human cell lines HEK293 and MRC5

Cell Line	**EC_{50} Value (nM)**
MCF7	7.99 ± 4.13
A549	0.188 ± 0.006
HEK293	>500
MRC5	>500

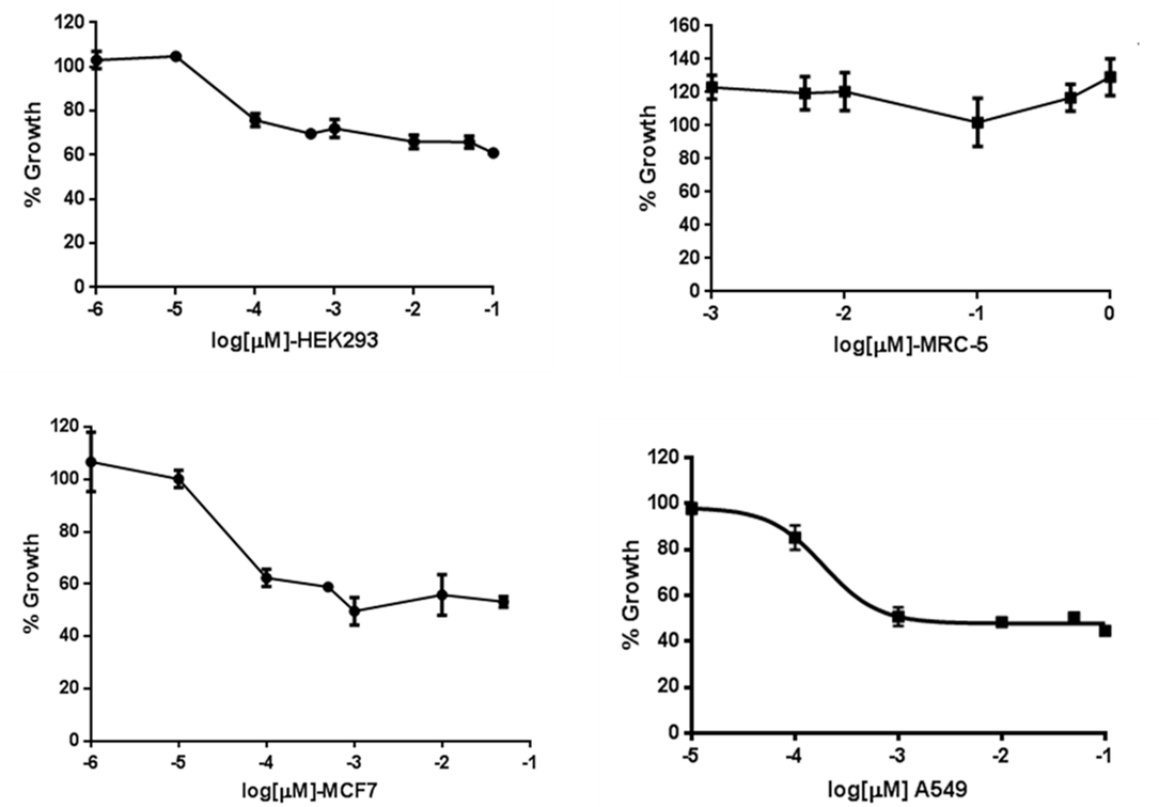

Figure 7 Dose-response curves for the indicated cell line in the presence of increasing concentrations of cruentaren A.

The observed selectivity is likely a consequence of the increased requirement that cancer cells have for ATP and Hsp90 chaperone activity compared to normal cells.[27] Transformed cells require higher concentrations of ATP than normal cells to maintain their elevated metabolic rate. In addition, the estrogen receptor and many other oncogenic clients are often overexpressed in cancer cells and rely upon the Hsp90 molecular chaperone for activity. Therefore, it was proposed that cruentaren A, a selective and potent inhibitor of F_1F_0 ATP synthase, which produces approximately 90 percent of the cells ATP, could decrease cellular levels of Hsp90-dependent client proteins and exhibit potent anti-proliferative activity against cancer cell lines with a high differential selectivity.[28]

Anti-proliferation studies using the known Hsp90 inhibitor geldanamycin co-dosed with fixed concentrations of cruentaren A were performed. No synergistic or additive activities were observed for the compounds, which may be due to the overexpression of Hsp90, as geldanamycin induces the heat shock response.

II.3 The cellular levels of Hsp90-dependent client proteins decreased after 48 hours of incubation with cruentaren A in a dose-dependent manner

Hsp90 inhibitors induce client protein degradation at concentrations that mirror their anti-proliferative IC_{50} value, as client protein degradation manifests a direct effect on cell growth. Similarly, cruentaren A exhibits an EC_{50} value of 7.99 ± 4.13 nM against MCF7 cells and client protein degradation was observed between 5-10 nM after 48 hour incubation. Cruentaren A also induced a dose-dependent decrease in the levels of Hsp90-dependent client proteins; phosphorylated Akt (pAkt), Her2 and Raf, which were undetectable at 50 nM (Figure 8). When compared to the vehicle control (0.25% DMSO), Hsp90 and F_1F_0 ATP synthase levels remained constant at both the 24 and 48 hour time points, whereas a modest decrease in Hsp70 levels was observed at 50 nM cruentaren A after 48 hours (Figure 8). Consistent with prior observations using efrapeptins, cruentaren A induced client protein degradation at low nanomolar concentrations without induction of Hsp70 and Hsp90. Levels of F_1F_0 ATP synthase were unchanged after 48 hours of incubation with cruentaren A, providing evidence that F_1F_0 ATP synthase is not an Hsp90-dependent substrate.

Incubation with 1 μM geldanamycin also resulted in client protein degradation at both 24 and 48 hours; however, dramatic increases in Hsp70 and Hsp90 levels were observed and indicate induction of the HSR, which is characteristic of geldanamycin and other Hsp90 N-

terminal inhibitors. This data indicates that cruentaren A functions as an inhibitor of the Hsp90 protein folding machinery and does not increase levels of Hsp70 and Hsp90, providing evidence that it does not bind Hsp90 in a manner similar to geldanamycin.

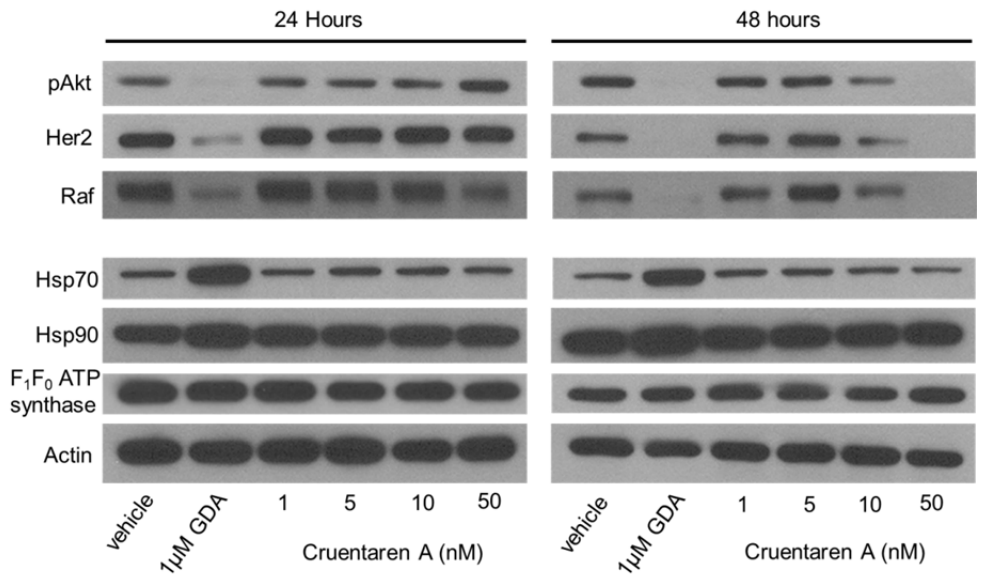

Figure 8 Western blot for Hsp90 client proteins (pAkt, Her2 and Raf) and Hsp70, Hsp90 and F_1F_0 ATP synthase using MCF7 cell lysates after 24 or 48 hours of incubation with the indicated amount of cruentaren A, vehicle (0.25% DMSO) or 1 μM geldanamycin (GDA).

II.4 Cruentaren A does not protect Hsp90 from trypsinolysis

Hsp90 inhibitors based on and including novobiocin bind the C-terminus and induce client protein degradation at concentrations that do not induce Hsp70 and Hsp90 levels (i.e. induce the HSR). Because cruentaren A also induced client protein degradation without increasing Hsp70 and Hsp90 levels, cruentaren A was suspected to bind and inhibit the Hsp90 C-terminus similar to novobiocin. To determine whether cruentaren A binds the Hsp90 C-terminus, trypsinolysis of Hsp90 in TnT rabbit reticulocyte in the presence of 50 nM and 50 μM cruentaren

A, a 1000-fold increase over the concentration needed to induce client protein degradation after 48 hours was investigated.[29, 30]

When Hsp90 is in the semi-closed and closed states, amino acids Lys615 and Arg620, are solvent exposed on an α helix and are susceptible to cleavage by trypsin. However, in the "extended" or "open conformation", these amino acids are protected and not subject to trypsinolysis.[30] Novobiocin binds the Hsp90 C-terminus and alters its conformational state by locking Hsp90 into the "open conformation", which prevents the cleavage of amino acids Lys615 and Arg620 and produces fragments that differ in molecular weight from the unprotected protein.[14]

In the presence of high concentrations of novobiocin, the C-terminal Hsp90 antibody AC88 detects the dose-dependent emergence of a 50 kDa band, while the N-terminal Hsp90 antibody identifies the disappearance of bands at 78 and 30 kDa, as well as doublet bands at 50 kDa and the emergence of a 73 kDa band (Figure 9A).[30] Both low and high concentrations of cruentaren A failed to protect Hsp90 from trypsinolysis and instead, exhibited proteolytic fingerprints identical to control (Figure 9B). The C-terminal Hsp90 antibody did not detect the presence of a 50 kDa band after incubation with cruentaren A. Likewise, the N-terminal Hsp90 antibody detected bands at 78 and 30 kDa, as well as the 50 kDa doublet bands and furthermore, did not detect the presence of a 73 kDa band following administration of cruentaren A. Consistent with prior studies, the N-terminal antibody detected bands at 40 and 22/24 kDa. These data indicate that cruentaren A does not bind the Hsp90 C-terminus and does not protect the C-terminus from trypsinolysis. In addition, incubation with cruentaren A did not produce a

proteolytic fingerprint that differed from vehicle control at low or high concentrations, which provides additional evidence that cruentaren A does not bind Hsp90.

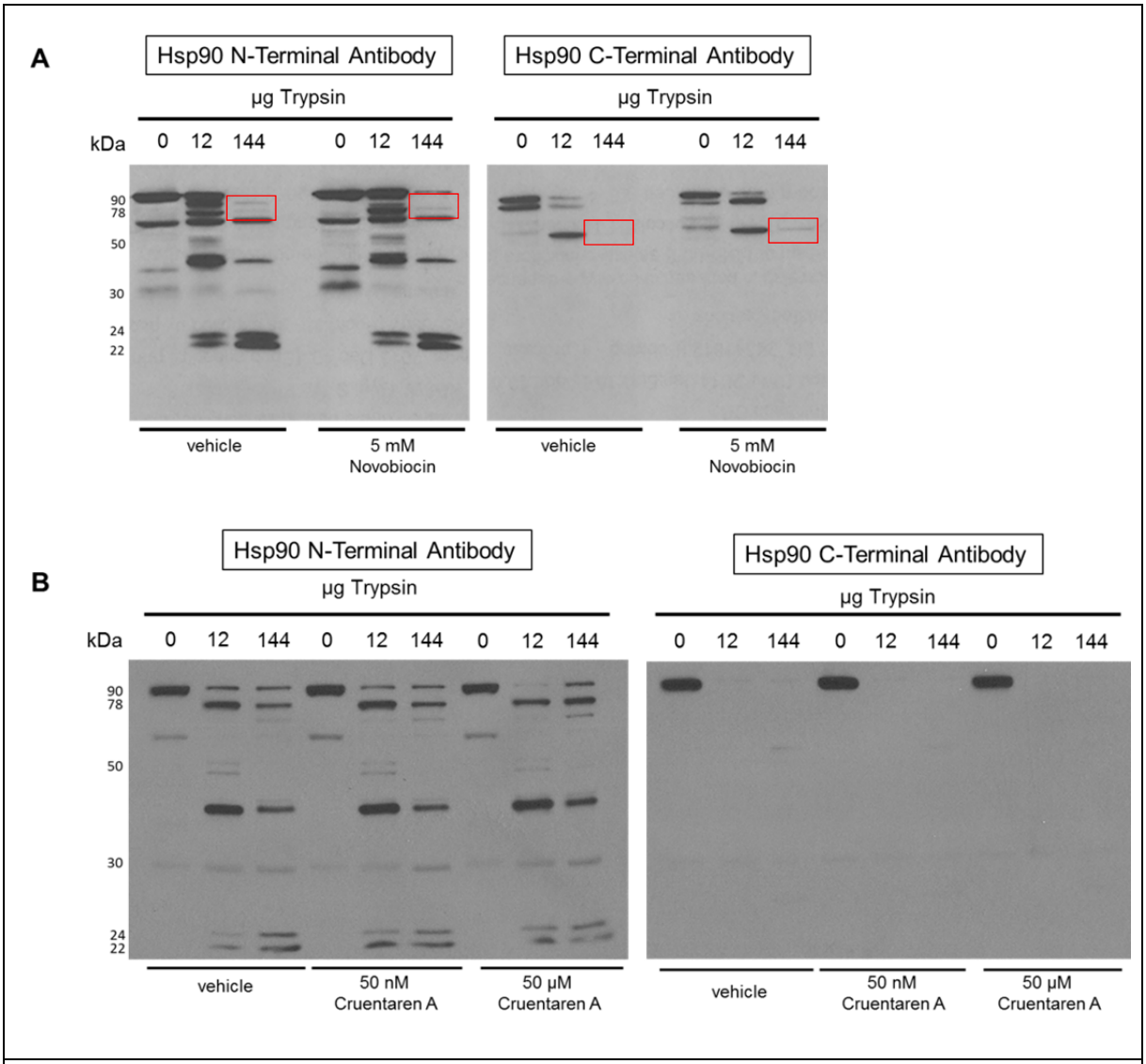

Figure 9 Proteolysis of Hsp90 from TnT reticulocyte lysate incubated under conditions of protein synthesis with (A) vehicle (1% DMSO) and 5mM novobiocin and (B) vehicle (1% DMSO), 50 nM or 50 µM cruentaren A. Antibodies specific to either the N- or C-terminus of Hsp90 were used to identify the Hsp90 fragments produced in the presence of increasing amount of trypsin.

II.5 Cruentaren A does not directly inhibit Hsp90 function

Firefly luciferase is an Hsp90-dependent substrate that produces bioluminescence upon the conversion of D-luciferin to oxyluciferin, and has been used to identify small molecule inhibitors of Hsp90 function.[31] Heat-denatured luciferase requires functional Hsp90 to re-fold and ultimately, produce bioluminescence. Monitoring bioluminescence over a range of concentrations indicates whether a molecule inhibits Hsp90 function in a dose-dependent manner. A cell-based luciferase re-folding assay has been used to characterize the Hsp90 C-terminal inhibitor, KU-174. KU-174 is a novobiocin analog that binds directly to the Hsp90 C-terminus, inducing client protein degradation and cell death at concentrations that do not activate the HSR.[13] The cell-based luciferase re-folding assay was used to determine whether cruentaren A inhibits Hsp90 function in a manner similar to KU-174 and/or geldanamycin.

Cruentaren A did not affect the re-maturation of luciferase up to a 1 μM concentration; however, both geldnamycin and KU-174 prevented the refolding of luciferase in a dose-dependent manner (Figure 10). This data suggests cruentaren A does not inhibit Hsp90 function, even at concentrations well above its EC_{50} value and at concentrations well beyond that needed to induce client protein degradation. When combined, these data suggest that cruentaren A does not bind or inhibit Hsp90, yet it induces client degradation at low nanomolar concentrations without induction of the HSR. Therefore, cruentaren A likely inhibits Hsp90 function by inhibiting F_1F_0 ATP synthase and provides evidence that F_1F_0 ATP synthase is important for modulation of the Hsp90 protein folding machinery.

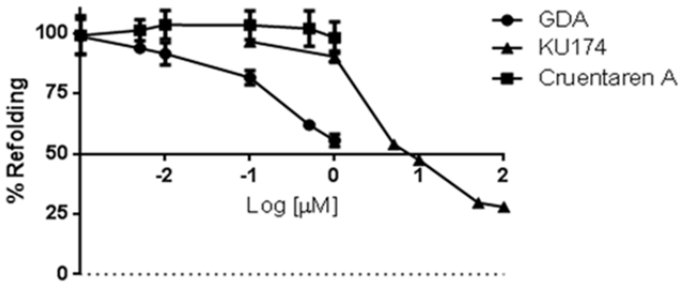

Figure 10 The percent of recovered luciferase activity after incubation of heat-denatured luciferase with geldanamycin (GDA), cruentaren A or KU174 compared to vehicle (1% DMSO). The concentrations of GDA and cruentaren A used during this assay ranged from 1 μM to 0.001 μM and the concentrations of KU174 ranged from 100 μM to 0.1 μM.

II.6 Interactions between Hsp90α and F_1F_0 ATP synthase is disrupted after 48 hours of incubation with cruentaren A, and Hsp90α exhibits altered cellular localization

Small molecules, such as celastrol and gedunin, disrupt the Hsp90 chaperone machinery by selectively targeting co-chaperone components of the heteroprotein complex.[17, 19, 32] Disrupting the heteroprotein complex prevents client protein maturation by preventing co-chaperone assistance during the chaperone cycle. In addition, the concentration needed to disrupt the Hsp90 protein folding machinery does not induce the HSR.

It has been shown that F_1F_0 ATP synthase interacts with Hsp90-client protein complexes in several cancer cell lines.[20] Inhibition of this interaction with efrapeptins not only disrupted interactions between F_1F_0 ATP synthase and Hsp90, but also destabilized the Hsp90-client protein complex, which resulted in client protein degradation via the ubiquitin-proteosome pathway.[20, 22] Furthermore, it was shown that geldanamycin exhibits no effect on the F_1F_0 ATP synthase-Hsp90 complex.[20]

As shown below, F_1F_0 ATP synthase directly interacts with Hsp90 in MCF7 cell lysates, specifically the Hsp90α isoform (Figure 11A and B). No other Hsp90 isoform was detected following co-immunoprecipitation with F_1F_0 ATP synthase using antibodies specific to Hsp90 isoforms, yet all Hsp90 isoforms were present. There also appears to be a cell surface population of F_1F_0 ATP synthase when immunostaining both permeable and impermeable MCF7 cells (Figure 11C). These data suggest the Hsp90α-F_1F_0 ATP synthase interactions may occur at the cell membrane, as Hsp90α is located in the cytosol.

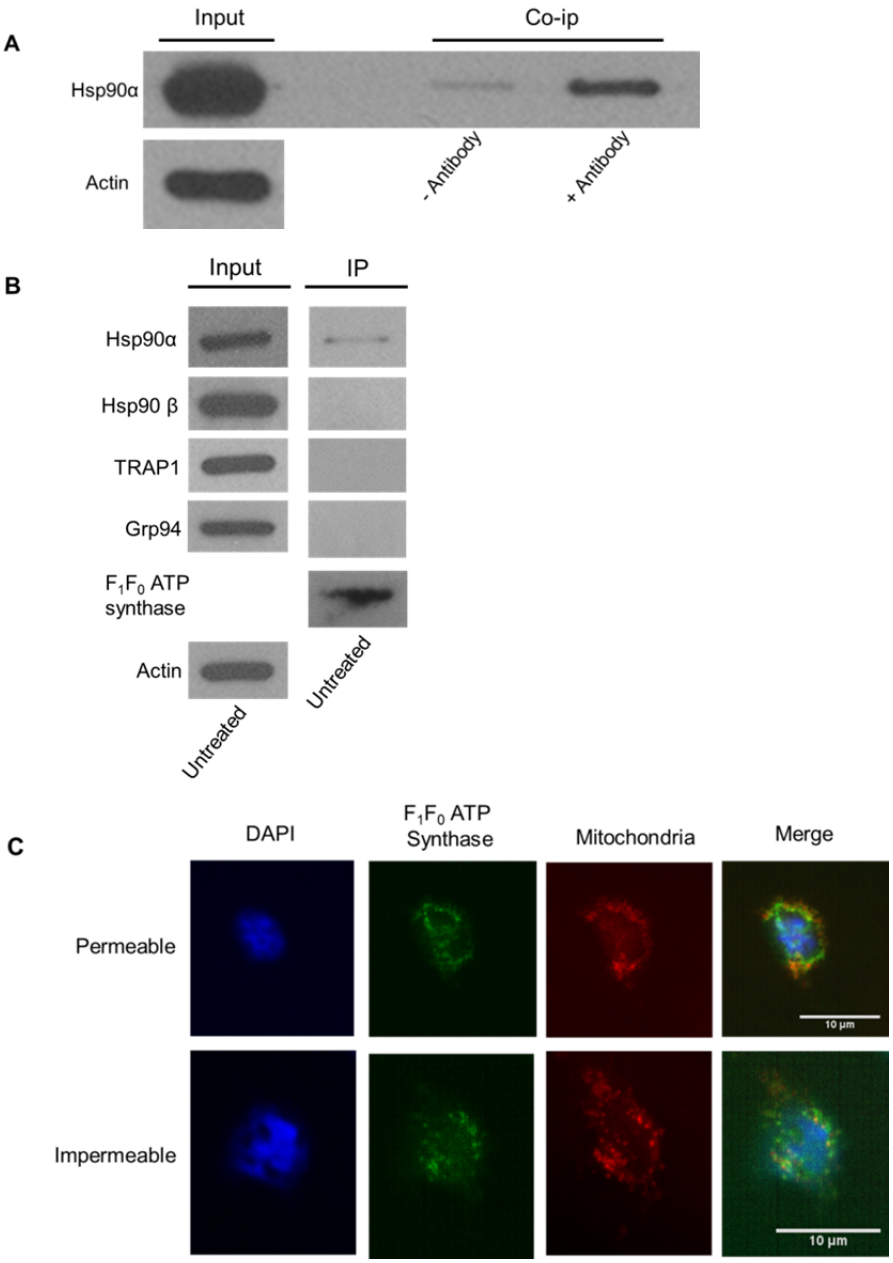

Figure 11 (A) Co-immunoprecipitation of Hsp90α with F_1F_0 ATP synthase from untreated MCF7 cell lysate with and without F_1F_0 ATP synthase-specific antibody. (B) Co-immunoprecipitation of Hsp90α using an anti-F_1F_0 ATP synthase antibody (Life Technologies) from untreated MCF7 cell lysate. (C) Immunofluorescence images of the nucleus (DAPI), F_1F_0 ATP synthase, the mitochondria, and the merged images of untreated permeable and impermeable MCF7 cells.

Interactions between F_1F_0 ATP synthase and Hsp90α remained unaffected after 24 hours of incubation with 50 nM cruentaren A; however, complete disruption was observed after 48 hours (Figure 12F). Cruentaren A also disrupted interactions between Hsp90α and F_1F_0 ATP synthase in a dose-dependent manner (Figure 12E).

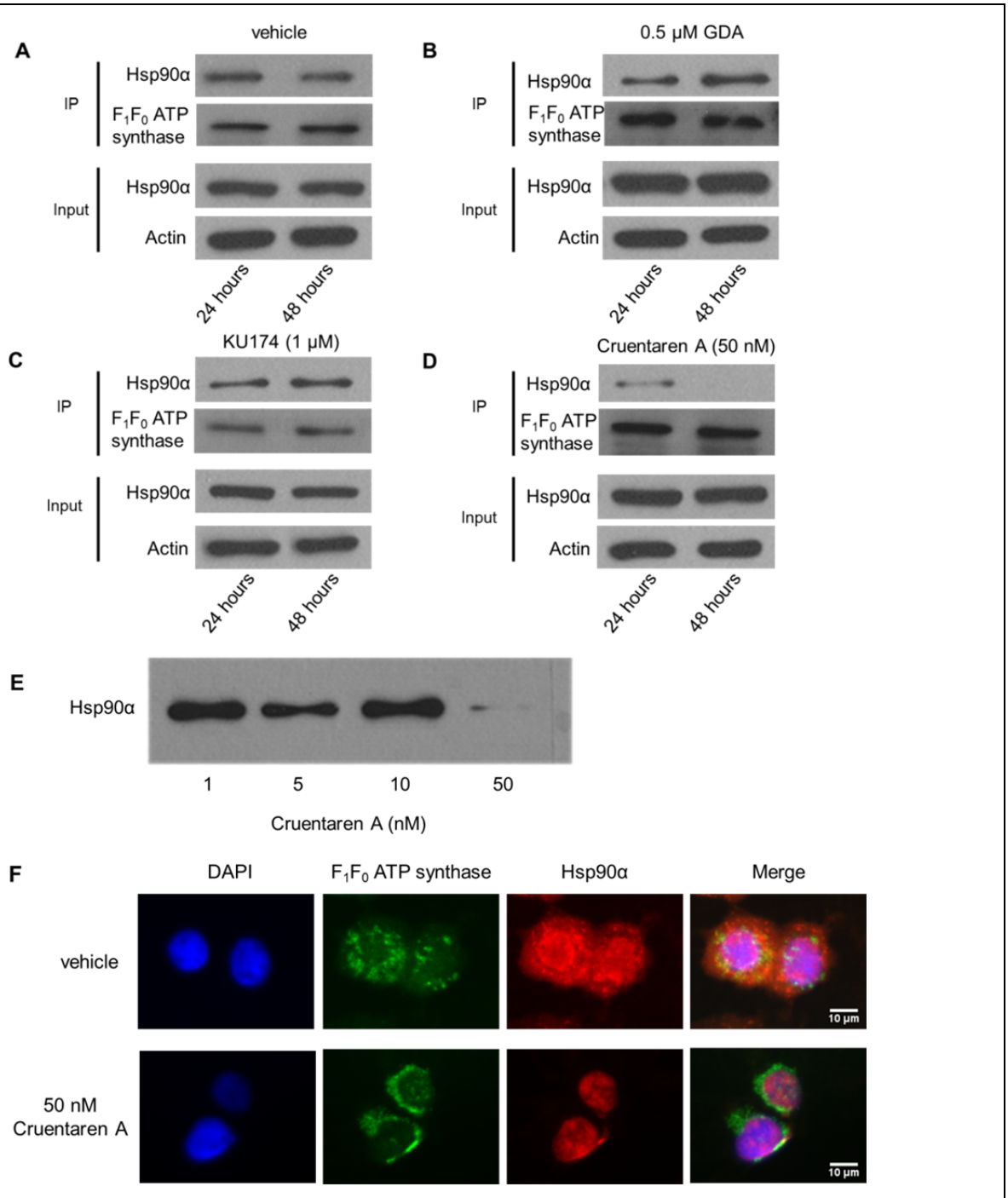

Figure 12 (A) Co-immunoprecipitation of Hsp90α using an anti-F_1F_0 ATP synthase antibody (Life Technologies) from MCF7 cell lysates treated for 24 and 48 hours with vehicle (0.25% DMSO), (B) 0.5 μM geldanamycin (GDA), (C) 1 μM KU174, (D) and 50 nM cruentaren A. (E) Dose-dependent disruption between Hsp90α and F_1F_0 ATP synthase after 48 hours of incubation with the indicated concentrations of cruentaren A. (F) Immunofluorescence images of the nucleus (DAPI), F_1F_0 ATP synthase, Hsp90α, and the merged images after 48 hours of incubation with either vehicle (0.1% DMSO) or 50 nM cruentaren A.

Interactions between Hsp90α and other components of the Hsp90 multiprotein complex (e. g. the Hsp90 co-chaperones Hsp70, Cdc37 and p23) were unaffected following 24 or 48 hours of incubation with cruentaren A (Figure 13).

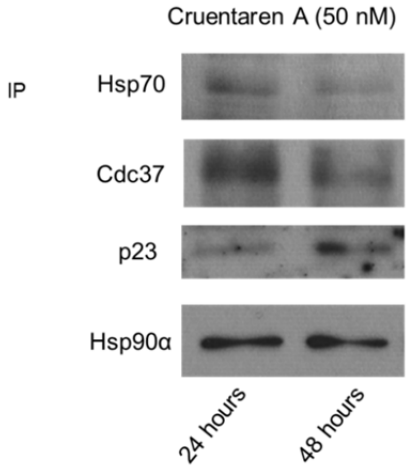

Figure 13 Western blot for the Hsp90 co-chaperones Hsp70, Cdc37 and p23 following co-immunoprecipitation using an anti-Hsp90α antibody (Assay Designs) from MCF7 cell lysates treated for 24 and 48 hours with 50 nM cruentaren A.

F_1F_0 ATP synthase-Hsp90α interactions also remained intact after 24 and 48 hours in the presence of a vehicle control, KU-174 and geldanamycin (Figure 12A, B and C). In contrast, interactions between F_1F_0 ATP synthase and Hsp90α increased in the presence of geldanamycin at 24 and 48 hours of incubation, which is likely a consequence of increased Hsp90α levels that result upon induction of the HSR. KU-174 had no effect on the F_1F_0 ATP synthase-Hsp90 interaction and co-immunoprecipitation results at 24 and 48 hours incubation were comparable to the vehicle control (Figure 12C). In addition, the cellular distribution of Hsp90α is dramatically

different after 48 hours of incubation with cruentaren A (Figure 12F). When comparing the immunofluorescence imaging of Hsp90α and F_1F_0 ATP synthase after 48 hours of incubation with 50 nM cruentaren A to vehicle control, Hsp90α translocates from an even distribution within the cytosol to localization at the nucleus. F_1F_0 ATP synthase distribution is slightly altered after 48 hours and exhibits increased localization around the nucleus.

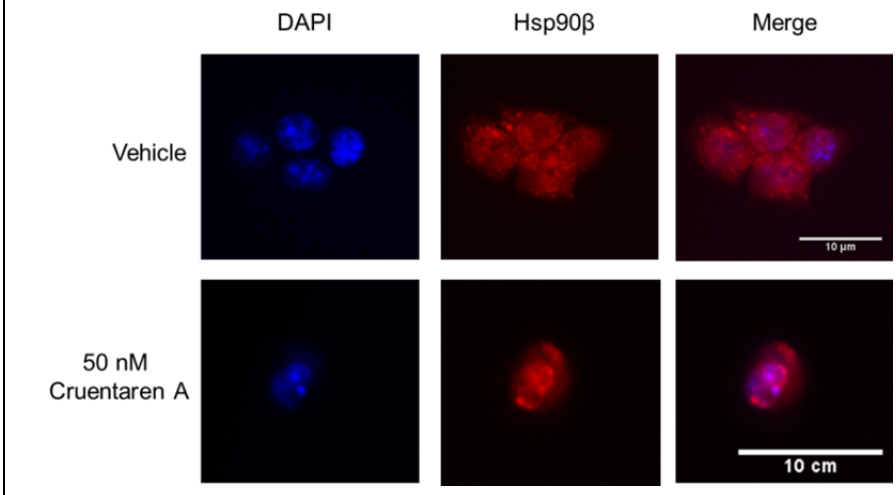

Figure 14 Immunofluorescence images of the nucleus (DAPI), Hsp90β, and the merged images of untreated MCF7 cells after 48 hours of incubation with either vehicle (0.1% DMSO) or 50 nM cruentaren A.

The other cytosolic Hsp90 isoform, Hsp90β, remained dispersed throughout the cytosol after 48 hours of incubation with 50 nM cruentaren A compared to vehicle (Figure 14). The cellular distribution of F_1F_0 ATP synthase and the Hsp90-dependent client protein, Raf-1, can be observed in Figure 15.

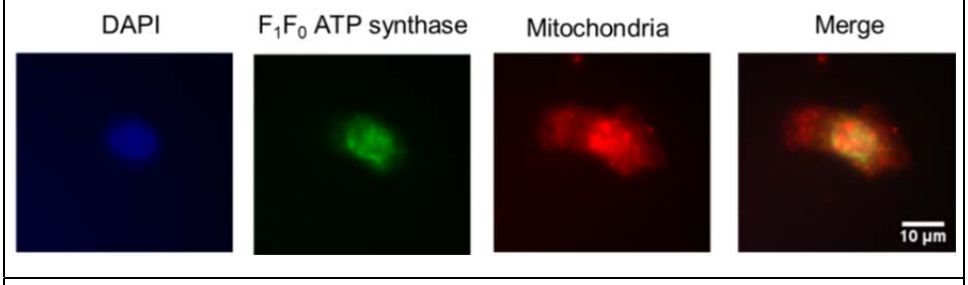

Figure 15 Immunofluorescence images of the nucleus (DAPI), F_1F_0 ATP synthase, Raf-1, and the merged images of untreated MCF7 cells.

Despite the apparent translocation of both Hsp90α and F_1F_0 ATP synthase towards the nucleus, there is a distinct difference in the cellular location of these proteins, which corroborates co-immunoprecipitation data that the interaction between Hsp90α and F_1F_0 ATP synthase is completely disrupted after 48 hours of incubation with 50 nM cruentaren A. Disruption of F_1F_0 ATP synthase and Hsp90α interactions resulted in a distinct cellular localization following 48 hours of incubation with cruentaren A, which also correlates directly with client protein degradation. Together, these data demonstrate the importance of functional F_1F_0 ATP synthase during client protein maturation and supports the potential of F_1F_0 ATP synthase as a target for disrupting Hsp90 function.

Due to Hsp90's involvement in multiple oncogenic pathways that contribute to the six hallmarks of cancer, inhibition of the Hsp90 chaperone machinery remains a promising strategy for the development of cancer chemotherapeutics despite the limitations of small molecule Hsp90 N-terminal inhibitors.[12] Alternative approaches to inhibiting Hsp90-dependent client maturation include disruption of interactions between Hsp90 and proteins that assist in client protein maturation during the chaperone cycle. Such small molecules include celastrol, which disrupts the interaction between Hsp90 and Cdc37, a co-chaperone that facilitates the maturation

of Hsp90-dependent kinases, and gedunin; which binds co-chaperone p23 and leads to Hsp90-p23 disruption and ultimately induces cancer cell death.[18, 19]

Papathanassiu, et. al. propose that F_1F_0 ATP synthase possesses co-chaperone function, as F_1F_0 ATP synthase directly interacts with Hsp90 and disruption of this interaction via efrapeptins prevents client protein maturation.[20, 22] Efrapeptins destabilize the interaction of Hsp90 with client proteins and ultimately induce client protein degradation through F_1F_0 ATP synthase inhibition; however efrapeptins are non-specific and are known to inhibit other ATP synthases and the 20S proteasome amongst others.[20, 22] Kunze, et. al. reported the small molecule cruentaren A not only inhibits F_1F_0 ATP synthase, but is selective for this ATP synthase over the Na^+/K^+ and V-type ATP synthases.[24] Cruentaren A also demonstrated selectivity for eukaryotic F_1 ATPases from yeast and mammals, but did not inhibit the function of F_1 ATPases from Escherichia coli.[24]

We have shown that cruentaren A indirectly causes Hsp90-dependent client degradation at low nanomolar concentrations through selective inhibition of F_1F_0 ATP synthase. Unlike Hsp90 N-terminal inhibitors, cruentaren A does not induce the pro-survival HSR upon client protein degradation. Hsp90 C-terminal inhibitors also avoid induction of the HSR while inducing client degradation; however, cruentaren A does not appear to bind the Hsp90 C-terminus at high concentrations nor did it directly inhibit Hsp90 function. It was demonstrated that F_1F_0 ATP synthase directly interacts with Hsp90 in MCF7 cell lysates and specifically interacts with the Hsp90α isoform. This interaction remained intact in the presence of the N-terminal inhibitor, geldanamycin, and the C-terminal inhibitor, KU-174, but was completely disrupted upon incubation with cruentaren A. Furthermore, the cellular distribution of Hsp90α and F_1F_0 ATP

synthase is dramatically altered upon incubation with cruentaren A. In the presence of vehicle, the cellular distribution of Hsp90α and F_1F_0 ATP synthase supports an interaction that occurs within the cytosol; however, upon incubation with cruentaren A, Hsp90α localizes to the nucleus while F_1F_0 ATP synthase remains relatively unaffected. Destabilization of the F_1F_0 ATP synthase-Hsp90α interaction and altered localization of these proteins correlates with client protein degradation upon incubation with cruentaren A. Therefore, selective inhibition of F_1F_0 ATP synthase results in client protein degradation by disrupting interactions between Hsp90α and F_1F_0 ATP synthase.

The hypothesis that ATP depletion affects the interaction between Hsp90 and client proteins has been suggested previously. Peng, et. al. observed that non-malignant myocytes treated with 2-deoxy-D-glucose or antimycin A, which are inhibitors of glycolysis and oxidative phosphorylation, respectively, disrupted interactions between Hsp90 and the client protein ErbB2, resulting in ErbB2 degradation.[33] It was shown that the mitochondrial ATP synthase inhibitor, oligomycin A, resulted in decreased levels of Her2 and pAkt after 48 hours of incubation comparable to cruentaren A; however, no degradation of Raf was observed and increased Hsp70 levels were observed with high concentrations of oligomycin A (Figure 16).

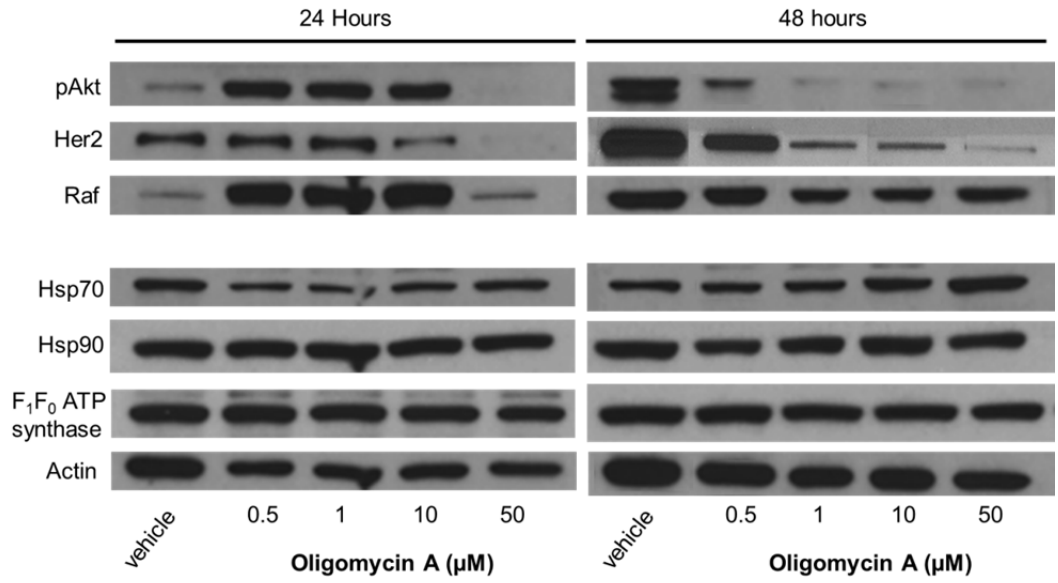

Figure 16 Western blot for Hsp90 client proteins (pAkt, Her2 and Raf) and Hsp70, Hsp90 and F_1F_0 ATP synthase using MCF7 cell lysates after 24 or 48 hours of incubation with the indicated amount of vehicle (0.25% DMSO) or oligomycin A. The calculated EC_{50} value of oligomycin A against the MCF7 cell line was 7.35 μM.

The mechanism by which cruentaren A inhibits F_1F_0 ATP synthase and whether or not it prevents proton translocation across the mitochondrial membrane, similar to oligomycin A, are not known.[34] Although treatment with cruentaren A or oligomycin A resulted in decreased levels of Her2 and pAkt after 48 hours of incubation, increased Hsp70 levels and unchanged Raf levels indicate oligomycin A and cruentaren A operate through related, but distinct mechanism(s) of action.

Collectively, these data suggest that Hsp90, specifically Hsp90α, functions as a cellular sensor of ATP and does so by directly interacting with F_1F_0 ATP synthase. Indeed, 80% of ATP from proliferating MCF7 cells occurs through oxidative phosphorylation.[35] This contradicts the Warburg hypothesis, which suggests malignancies switch from oxidative ATP production to

glycolytic; however, numerous studies have found this to be dependent upon the individual cancer and the cancer cell environment.[35-37] As previously mentioned, numerous other cancer cells, including the MCF7 cell line, have an increased concentration of Hsp90α and elevated chaperone activity. Given the ATPase activity of Hsp90, it is plausible that Hsp90 functions as a cellular sensor of ATP and that Hsp90α, the most abundant Hsp90 isoform in cancer cells, requires a significant amount of ATP and therefore directly interacts with the cell's source of ATP, F_1F_0 ATP synthase. Depriving Hsp90α of ATP by inhibiting F_1F_0 ATP synthase exhibits a debilitating effect on the chaperone cycle that utilizes oxidative phosphorylation as their ATP source. Simultaneously inhibiting ATP production and Hsp90 chaperone activity may explain the potency of cruentaren A among the many different cancer cell lines and provides a novel approach to modulate the Hsp90 protein folding machinery.

Therefore, inhibition of F_1F_0 ATP synthase via cruentaren A and disruption of the interaction between Hsp90α and F_1F_0 ATP synthase represents a novel and powerful approach toward inhibiting client protein maturation devoid of the HSR. While work remains to be done, cruentaren A represents a new class of Hsp90 modulators that targets the Hsp90α-F_1F_0 ATP synthase complex and represents a new paradigm to modulate the Hsp90 protein folding machinery.

II.7 Methods and Experimentals

Antibodies and Reagents

Antibodies targeting Hsp90β, Grp94, Raf-1 and actin were purchased from Santa Cruz Biotechnology. Antibodies targeting Hsp90α-2 and Hsp70 were purchased from Assay Designs. Antibodies targeting Cdc37, p23, and an additional Raf-1 antibody were purchased from abcam.

The remaining antibodies are listed and were purchased from the indicated vendors: TRAP1 (BD Biosciences); pAKT (Cell Signaling); Her2 (c-erB-2) (Invitrogen); F_1F_0 ATP synthase subunit β (Life Technologies and proteintech). The antibody targeting the N-terminus of Hsp90 was purchased from Thermo Scientific (PA3-013) and the antibody targeting the C-terminus Hsp90 was purchased from Enzo Life Sciences (AC88). KU174 and cruentaren A were synthesized in house and geldanamycin was purchased from Sigma Aldrich[38, 39].

Cell Culture

The media for each cell line was supplemented with streptomycin (500 μg mL^{-1}), penicillin (100 units mL^{-1}), and 10% FBS. MCF7 cells were maintained in Advanced DMEM/F12 (1:1; Gibco) supplemented with L-glutamine (2 mM). A549 cells were maintained in F12K (Cellgro). MRC-5 cells were maintained in DMEM (Cellgro). Wild type and luciferase-expressing PC3-MM2 cells (a gift from George Vielhauer) were maintained in MEME (Sigma) supplemented with 5 μg mL^{-1} puromycin. Cells were grown in a humidified atmosphere (37 °C, 5% CO_2) and passaged when confluent.

Anti-proliferation

Cells were grown to confluence, seeded (2000 cells/well, 100 μL total media) in clear, flat-bottom 96-well plates and allowed to attach overnight. Compound or geldanamycin at varying concentrations in DMSO (1% DMSO final concentration) was added. Cells were returned to the incubator for an additional 72 h. After 72 h, cell growth was determined using an MTS/PMS cell proliferation kit (Promega) per the manufacturer's instructions. Cells that incubated in 1% DMSO were used as 100% proliferation (i.e. DMSO = 100% growth) and the relative growth for

each compound concentration was compared to 1% DMSO. IC50 values were calculated from two separate experiments performed in triplicate using GraphPad Prism 6.0.

Western Blot

MCF7 cells were grown to confluence and seeded at 0.4 x 10^6 cells/well/2 mL. Cells were incubated for 24 hours and treated with varying concentrations of cruentaren A or 1 μM geldanamycin in DMSO (0.25% DMSO final concentration), or vehicle (DMSO) for 24 or 48 hours. Cells were harvested in cold PBS and lysed using MPER (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) according to manufacturer's directions. Lysates were clarified at 14,000g for 15 minutes at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (5 μg) were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with the corresponding antibody. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Proteolytic Fingerprinting Assay

Rabbit reticulocyte (Green Hectares) incubated under conditions of protein synthesis at 30^{o}C in the presence of compound or vehicle (1% DMSO) for 10 minutes. Each reaction mixture contained 66.6% rabbit reticulocyte and 33.3% ATP regenerating system (10 mM creatine phosphate and 20 μg mL^{-1} creatine phosphokinase) and a final concentration of 75 mM KCl. Each reaction mixture contained the indicated amount of compound. After incubating, the samples were immediately placed on ice and the indicated amount of TPCK-treated trypsin (Worthington) was added to each sample. The samples digested on ice for an additional 6

minutes and the reactions were quenched by the addition of Laemmli sample buffer followed by immediate boiling. Equal amounts of each sample were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with antibodies specific to the N-terminus of Hsp90 or the C-terminus of Hsp90. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Luciferase Re-folding Assay

Compound at varying concentrations in DMSO (1% DMSO final concentration) was added to wells of a white, round-bottom 96-well plate containing 50 μL of MEME media. Luciferase-expressing PC3-MM2 cells were grown to confluence, collected and incubated for 8- 12 minutes at 50^{o}C in pre-warmed MEME media until bioluminescence of luciferase was reduced to 1% of the initial counts. Cells were added (60,000 cells/50 μL) to wells (final concentration of 60,000 cells/100 μL) and the plate was returned to the incubator for 1 hour. After 1 hour, 100 μL of luciferase substrate reagent (75 mM tricine at pH7.8, 24 mM $MgSO_4$, 0.3 mM EDTA, 2 mM DTT, 0.313 D-luciferin, 0.64 mM co-enzyme A, 0.66 mM ATP, 150 mM KCl, 10% Triton-X, 20% glycerol and 3.5% DMSO) was added to wells and the bioluminescence was immediately read (0.5 second integration time). Cells that incubated in 1% DMSO were used as 100% bioluminescence (i.e. DMSO = 100% re-folding) and the relative re-folding for each compound concentration was compared to 1% DMSO. The concentrations for each compound were in triplicate and dose-response curves were generated using GraphPad Prism 6.0.

Co-immunoprecipitation

MCF7 cells were grown to confluence and seeded at $2x10^6$ cells/5 mL in 10 cm dishes. Cells were incubated for 24 hours and then treated with either 0.5 μM geldanamycin, 1 μM KU174 or 50 nM cruentaren A in DMSO (0.25% DMSO final concentration), or vehicle (DMSO) for the indicated lengths of time. Media and cells were collected with PBS and centrifuged at 200g for 5 minutes at 4^oC. Supernatant was aspirated and pellets were washed one time with cold PBS and centrifuged. Supernatant was aspirated and cell pellets were subsequently suspended in the non-denaturing lysis buffer (10 mM Tris-HCl at pH 7.5 and 0.2% NP-40 (v/v)) and incubated on ice for 2 hours. Lysates were clarified at 14,000g for 15 min at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal protein (400 or 500 μg) was incubated with 1 μg of anti-F_1F_0 ATP synthase antibody in 500 μL total volume lysis buffer for approximately 16 hours with rocking at 4 °C. Following incubation, 30 μL of re-suspended Dynabeads Protein A (Invitrogen) was added and incubated with rocking for 1 hour at 4 °C. Protein A beads were washed 3 times with lysis buffer (500 μL) and suspended in Laemmli sample buffer (15 μL) and were boiled for 15 minutes to dissociate proteins from beads. Samples were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with the indicated antibodies. Membranes were incubated with a species-appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Immunofluorescence Analysis

MCF7 cells were grown to confluence, seeded (1000 cells/well, 100 μL total media) in black/clear well, flat-bottom 96-well plates and allowed to attach overnight. Cruentaren A in DMSO (0.1% DMSO final concentration) was added and incubated at 48 hours. Cells were

washed with PBS and fixed with freshly made 4% (w/v) paraformaldehyde in PBS for 25 minutes, washed with PBS, permeabilized with 0.1% (v/v) Tween-20 in PBS for 5 minutes, washed with PBS, blocked with 3% (w/v) BSA in PBS for 1 hour, washed with PBS, and incubated with primary antibody targeting F_1F_0 ATP synthase subunit β and/or Hsp90α-2 at a 1:200 and a 1:400 concentration, respectively, in 3% BSA in PBS at 4°C overnight. The cells were then washed with PBS, incubated with secondary antibody conjugated with Alexa Fluor 488 or 568 for 3 hours at 4°C, washed with PBS, and counterstained the DNA with DAPI. Cells that underwent mitochondrial staining incubated with MitoTracker® Red CMXRos prior to fixing according to the manufacturer's instructions. Confocal images were acquired sequentially with SlideBook Version 5.0 software on a 3I Spinning Disk Confocal Inverted Microscope (Olympus) using 40x long working distance air lenses. Images were processed using Image J software (NIH).

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Chapter III

Natural Products that Disrupt Hsp90-dependent Client Protein Maturation

III.1 Disruption of Hsp90-co-chaperone interactions

Hsp90 is a molecular chaperone that is responsible for the conformational maturation of many newly synthesized polypeptides and the re-maturation of denatured proteins termed "clients" via the Hsp90 chaperone cycle. Hsp90 functions as a homodimer and requires the formation of the heteroprotein complex that is composed of immunophilins, co-chaperones and partner proteins for assistance during chaperone activity. Several conformational transitions of the Hsp90 heteroprotein-client complex, coupled to Hsp90 ATPase activity results in folding and release of the client protein substrate.[1, 2] Inhibition of the chaperone cycle leads to client protein ubiquiltinylation and subsequent degradation via the proteasome.[3-5] Hsp90 inhibition has emerged as a strategy for anticancer chemotherapeutic development due to the involvement Hsp90-dependent clients in a variety of oncogenic signaling pathways.[6-11] Oncogenic client degradation via Hsp90 inhibition ultimately halts cancer progression and has been observed during clinical trials with Hsp90 inhibitors.

Classic small molecule Hsp90 inhibitors are designed to perturb N-terminal ATPase activity and include derivatives of geldanamycin (**1**) (Figure 17), radiciol and several purine analogs.[12] Although Hsp90 N-terminal inhibitors are effective at inhibiting Hsp90 function and lead to tumor digression through client degradation, N-terminal inhibition also leads to displacement of the Hsp90-bound transcription factor, Heat Shock Factor-1 (HSF-1) and induction of the pro-survival heat shock response (HSR).[13-16] Upon displacement, HSF-1 trimerizes, translocates to the nucleus and induces transcription of various heat shock proteins,

including Hsp90. Unfortunately, such induction has led to issues with dosing and scheduling during clinical trials with N-terminal inhibitors. Therefore, anticancer Hsp90 inhibitors that avoid induction of the HSR are needed.

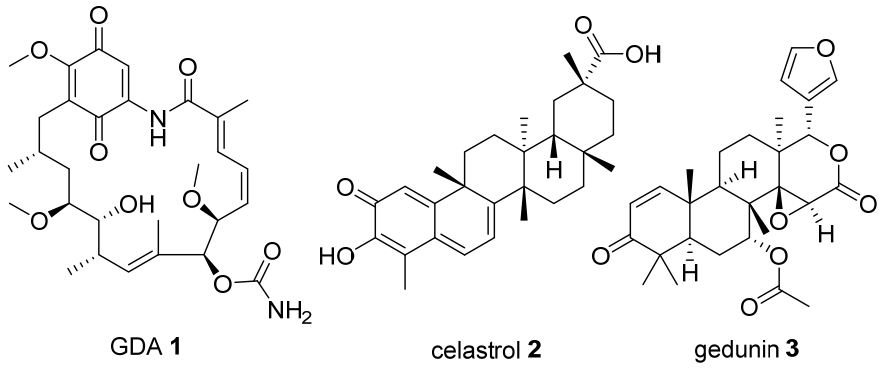


Figure 17 Structures of geldanamycin (GDA) (**1**), celastrol (**2**) and gedunin (**3**).

Alternative strategies to inhibit the chaperone cycle include targeting the Hsp90 C-terminus and disruption of the heteroprotein complex. C-terminal inhibitors include derivatives of the coumarin-containing natural product, novobiocin.[17-20] These inhibitors prevent cancer cell proliferation at concentrations similar to N-terminal inhibitors and destabilize Hsp90-client protein interactions without induction of the HSR. Maturation of client protein substrates also require additional proteins that interact with Hsp90 and modulate ATPase activity and/or assist in client folding.[21, 22] For example, the co-chaperone, Hsp90-Hsp70 organizing protein (HOP), associates with the Hsp90 dimer during the initial stages of the chaperone cycle and facilitates the delivery of certain clients from Hsp70 to Hsp90. Peptidyl-prolyl isomerase co-chaperones (PPIases) assists in the folding and 3-dimensional maturation of clients, and the co-chaperone p23 promotes the Hsp90 conformation optimal for ATP binding and hydrolysis, which ultimately results in client protein release.[23] In addition, some substrates require the assistance of specific

co-chaperones for maturation (e.g. all Hsp90-dependent kinases require the assistance of the co-chaperone cell division cycle 37 (Cdc37) for maturation). Disruption of interactions between Hsp90 and co-chaperones may allow for the select degradation of clients and avoid systemic client protein degradation, a consequence of Hsp90 inhibitors currently under clinical investigation and may be responsible for deleterious side effects.

The cucurbitacin class of natural products is a group of triterpenoids that are present in the Cucurbitaceae family and other closely related families. More than 50 cucurbitacins have been identified and exhibit a wide variety of biological activities that include, but not limited to, cytotoxicity, anti-proliferative and anti-inflammatory activity, anti-oxidant, anti-hepatotoxicity, anti-bacterial and anti-viral properties, as well as anti-metastatic and improved anticancer activity when combined with current chemotherapies.[24-26] Despite the presence of an α-,β-unsaturated ketone (Michael acceptor; a well-established and non-specific disruptor of general cellular processes via electrophilic chemical reactivity) located within many of the cucurbitacin compounds, several cucurbitacins interact with specific cellular targets involved in gene regulation, transcription and overall cellular fate. Cucurbitacin D (**4**) reduced the proliferation of T-cell leukemia cells and ultimately led to cancer cell apoptosis.[27] This cucurbitacin was shown to affect the NF-κB pathway and lead to the accumulation of NF-κB and IκB-α within the cytosol. Interestingly, a decrease in cellular levels of Bcl-xL and Bcl-2 was also observed. Bcl-xL and Bcl-2 levels are regulated by Hsp90 clients that depend upon the chaperone cycle for folding and function. It has been reported that incubation with N-terminal inhibitors led to cellular decreases in Bcl-xL and Bcl-2 levels.[28, 29] Furthermore, **4**, as well as other cucurbitacins, share structural similarities to the natural products, celastrol (**2**) and gedunin (**3**), which are known inhibitors of client maturation via disruption of the Hsp90 heteroprotein complex (Figure

17). However, these natural products inhibit chaperone function via different mechanisms. **2** covalently binds the Hsp90 N-terminus and disrupts interactions between Hsp90 and Cdc37 to ultimately result in kinase client degradation.[30] **3** binds the co-chapreone p23 and disrupts Hsp90-co-chaperone interactions, leading to client degradation across many different cancer cell lines.[31] Given the structural similarities between the cucurbitacins, **2** and **3**, as well as decreased levels of proteins that depend upon functional clients after exposure to cucurbitacins, it was hypothesized that **4**, and other cucurbitains, may inhibit the Hsp90 chaperone cycle in a manner similar to **2** and/or **3**.

In line with our hypothesis, select cucurbitacins prevented Hsp90-dependent client protein maturation: compound **4** (cucurbitacin D) and 3-*epi*-isocurbitacin D (**6**).[32] **4** prevented the maturation of Hsp90-dependent clients by disruption of the Hsp90 heteroprotein complex[32]. With the exception of 23,24-dihydrocucurbitacin D (**9**) and 23,24-dihydrocucurbitacin B (**10**), all cucurbitacins exhibited anti-proliferative IC_{50} values at nanomolar concentrations against the MCF7 breast cancer cell line[32]. Western blot analysis for client levels in the presence of high and low concentrations of each cucurbitacin revealed that incubation with **4** and **6** led to client degradation[32]. **4** also disrupted the interaction between Hsp90 and the co-chaperones Cdc37 and p23; however, **6** did not disrupt these Hsp90-co-chaperone interactions and caused client degradation through alternative mechanism(s)[32]. Furthermore, **4** did not induce the HSR, as no increases in the levels of heat shock proteins Hsp90, Hsp70 or Hsp27 was observed[32]. Client protein degradation, disruption of Hsp90-Cdc37 and Hsp90-p23 interactions as well as a lack of heat shock protein induction, notably Hsp27, has previously been observed after incubation with **3** and suggested **4** inhibited cancer cell growth through Hsp90 client degradation via disruption of Hsp90-co-chaperone interactions by a mechanism comparable to **3**.

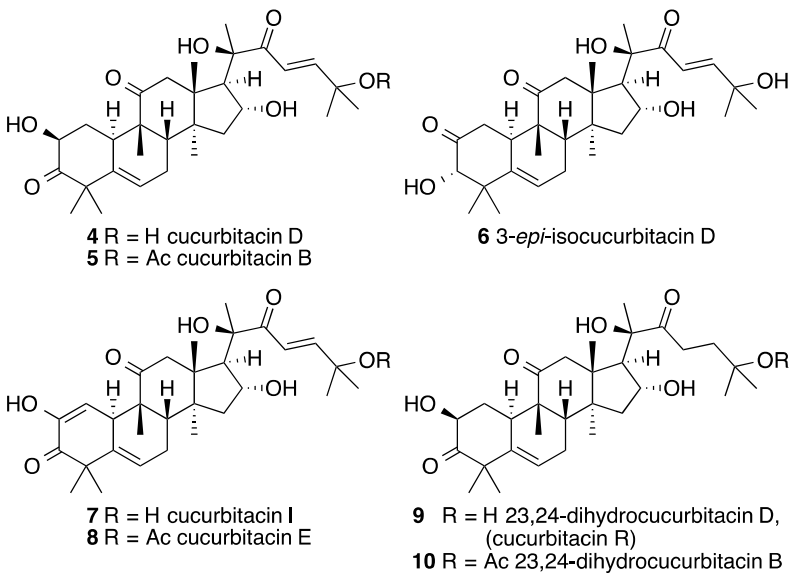


Figure 18 Chemical structures of cucurbitacins isolated from *Cucurbita texana* (Cucurbitaceae) [cucurbitacin D (**4**) and B (**5**); 3-*epi*-isocucurbitacin D (**6**); cucurbitacin I (**7**) and E (**8**)] and the structures of the semi-synthesized natural products [3-*epi*-cucurbitacin D (**6**), cucurbitacin I (**7**), 23,24-dihydrocucurbitacin D (**9**) (cucurbitacin R) and B (**10**)].

III.2 Isolation and semi-syntheses of the cucurbitacins

All cucurbitacins within this chapter were isolated and/or semi-synthesized in the laboratory of Dr. Fathi Halaweish at South Dakota State University. Structures of the isolated and semi-synthesized cucurbitacins of interest are shown in Figure 18. A brief description of the methods used to produce these compounds is provided. Detailed experimental procedures can be found at the end of this chapter.

Cucurbitacin D (**4**) and cucurbitacin B (**5**), as well as isomeric 3-*epi*-isocucurbitacin D (**6**) were isolated from the fruits of *Cucurbita texana* (Cucurbitaceae) utilizing previously described procedures.[33-35] Two additional oxidized derivatives, cucurbitacin I (**7**) and E (**8**) were also obtained. Compounds **7** and **8**, along with **6**, were obtained in reduced quantities compared to **4**

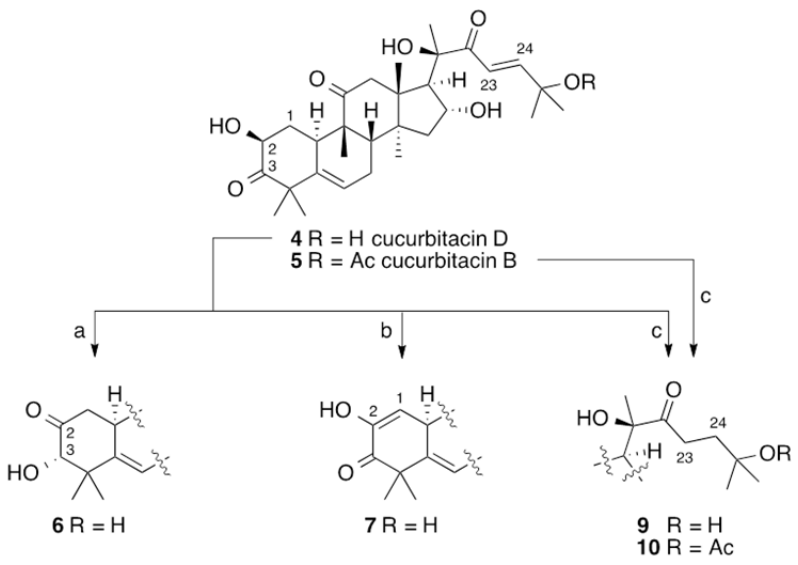
and **5**. Fortunately, there is literature precedence to semi-synthetic approaches for these particular compounds; these approaches were a solution to the lack of material for these natural products. Furthermore, **9**, **10** and other reduced C23-24 derivatives have been routinely acquired via hydrogenation conditions**.**

Investigation by Galindo and co-workers has determined the isomerization of the α-hydroxyketone at C2 and C3 of select cucurbitacins takes place under both acidic and basic conditions.[36, 37] However, a similar alternative procedure to Galindo's was selected, which was similar to that reported by Sneden et al.[38] This procedure takes advantage of the slightly acidic nature of silica gel. Heating a CH_2Cl_2 solution of **4** and silica gel to 40 ºC provided sufficient quantities of **6** for biological evaluation with the advantage of recovering unreacted **4** (Scheme 1).

During attempts to improve the biological activity of cucurbitacins through formation of cucurbitacin derivatives via the Heck reaction[39] an unexpected product was isolated and later determined by spectroscopic analysis to be **7**. Although this is not the first report of the oxidation of the α-hydroxyketone to a diosphenol moiety for cucurbitacins,[40-42] this is the first example of this transformation via a Saegusa oxidation (Scheme 1).[43-46] This result can be rationalized by the ability of cucurbitacins to isomerize and form the intermediate enediol, which can be trapped and oxidized to the final diosphenol product. This enediol is the same intermediate that is required for the formation of isocucurbitacins. While **7** was not the desired product, the resulting material was suitable for biological testing.

To date, *Cucurbita texana* has not produced **9** or **10** via natural product isolation. However, hydrogenation of the C23-24 olefin of **4** and **5** has facilitated the ability to obtain

useful quantities of semi-synthesized compounds **9** and **10** from plant species that do not routinely provide them through natural product isolation (Scheme 1).[36, 40, 47-53]



Scheme 1 Semi-synthesis of cucurbitacins: 3-*epi*-isocucurbitacin D (**6**), cucurbitacin I (**7**), 23,24-dihydrocucurbitacin D (**9**) (cucurbitacin R) and 23,24-dihydrocucurbitacin B (**10**). *Reagents and conditions*: a) silica gel, CH_2Cl_2, 40 ºC, 25% (30% conversion); b) $Pd(OAc)_2$, PhBr, NaOAc, DMA, 80 ºC, 38% (60% conversion); c) H_2 (1atm), Pd/C 5%, EtOH, 88%.

III.3 Select cucurbitacins exhibit potent anti-proliferative activity and decrease Hsp90-dependent client protein levels without induction of the HSR

Consistent with previous data against several cancer cell lines, many of these cucurbitacins exhibited nanomolar IC_{50} values against MCF7 breast cancer cell line proliferation. The decrease in potency observed for **9** and **10** may be explained by reduction of the α-, β-unsaturated ketone. This would reduce the potential for non-specific, attack by cellular nucleophiles. The IC_{50} values for each cucurbitacin can be found in Table 5 along with values for **1**, an Hsp90 N-terminal inhibitor, **2** and **3**.

Table 5 Calculated IC_{50} values for the controls geldanamycin (GDA) (**1**), celastrol (**2**), gedunin (**3**) and select cucurbitacins against MCF7 breast cancer cell line proliferation.

Compounds	**MCF7 IC_{50} Values (μM)**
GDA (**1**)	0.0427 ± 0.018
celastrol (**2**)	0.153 ± 0.004
gedunin (**3**)	10.9 ± 0.9
cucurbitacin D (**4**)	0.598 ± 0.001
cucurbitacin B (**5**)	0.0413 ± 0.003
3-*epi*-isocucurbitacin D (**6**)	0.718 ± 0.009
cucurbitacin I (**7**)	0.0607 ± 0.012
cucurbitacin E (**8**)	0.055 ± 0.006
23,24-dihydrocucurbitacin D (**9**)	5.13 ± 0.01
23,24-dihydrocucurbitacin B (**10**)	46.3 ± 0.1

Hsp90 inhibitors and disruptors of the heteroprotein complex prevent cancer cell proliferation at concentrations near the IC_{50} value by depleting levels of clients necessary for cancer cell growth. To correlate client degradation via inhibition of the chaperone cycle with the IC_{50} value for each cucurbitacin, client levels from MCF7 cell lysates dosed with high and low concentrations (5-times the IC_{50} value and one-half the IC_{50} value, respectively) of each cucurbitacin were detected by Western blot analysis. The levels of heat shock proteins were also detected to monitor for induction of the HSR as well as levels of the co-chaperones, p23 and Cdc37. Incubation with high concentrations of the control compounds **1**, **2** and **3** led to client

protein degradation and little change in the levels of Cdc37 and p23 (Figure 19B). Consistent with previous reports, incubation with GDA led to increased levels of Hsp90, Hsp70 and Hsp27, indicating induction of the HSR (Figure 19B). Minimal induction of the HSR was observed at the concentrations of **2** and **3** evaluated; while incubation with **3** resulted in a slight increase in Hsp70 levels alongside comparable levels of Hsp90 and Hsp27 to vehicle control upon incubation with **2** and **3** (Figure 19B).

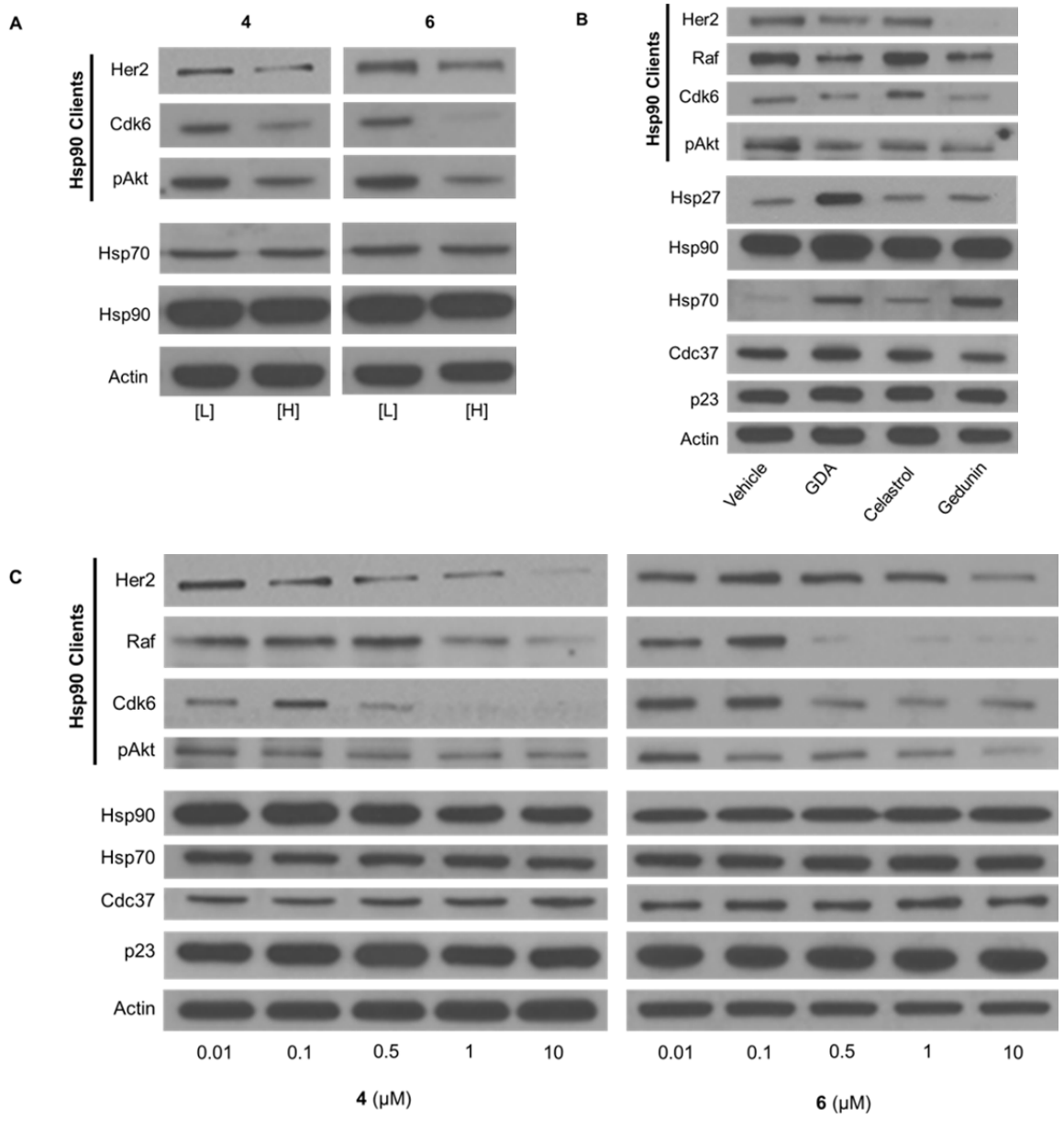


Figure 19 (A) Western blot for Hsp90 client proteins (pAkt, Her2 and Cdk6) and heat shock proteins (Hsp70 and Hsp90) from cell lysates dosed for 24 hours with high and low concentrations (five times the IC_{50} value and half the IC_{50} value, respectively) of the cucurbitacin D (**4**) and 3-epi-isocucurbitacin D (**6**) (B) Levels of Hsp90 clients (pAkt, Her2, Cdk6 and Raf), heat shock proteins (Hsp90, Hsp70 and Hsp27) co-chaperones (Cdc37 and p23) and actin from MCF7 cell lysates treated for 24 hours with vehicle (0.25% DMSO) or high concentrations (5-times the IC_{50} value) of the controls geldanamycin (GDA) (**1**), celastrol (**2**), gedunin (**3**) (C) Levels of Hsp90 clients, heat shock proteins and co-chaperones from cell lysates treated with vehicle or increasing concentrations of cucurbitacin D (**4)** or 3-*epi*-isocucurbitacin D (**6**).

Levels of clients and heat shock proteins after incubation with high and low concentrations of each cucurbitacin can be found in Figure 19A and in Figures 20 and 21.

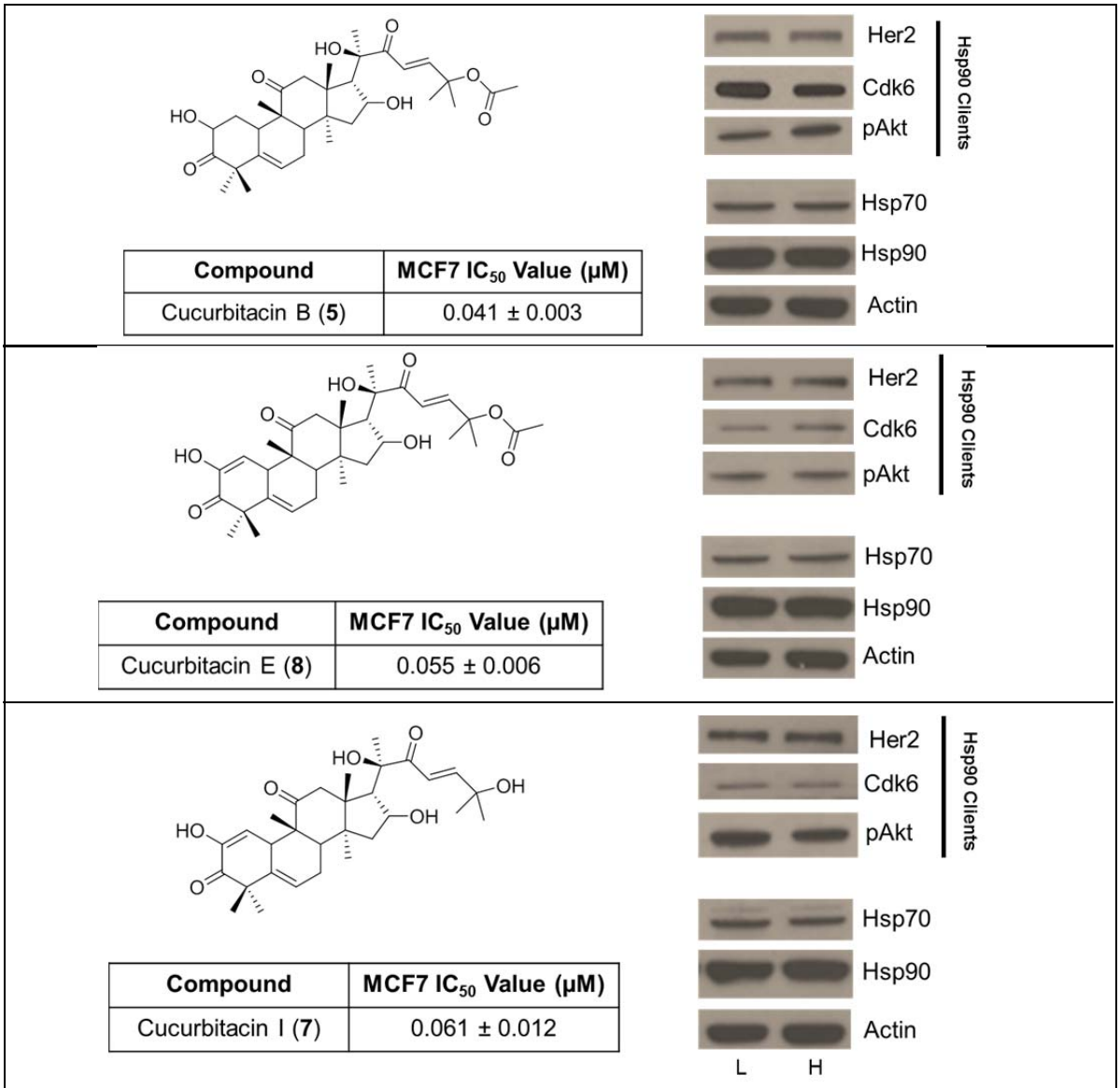
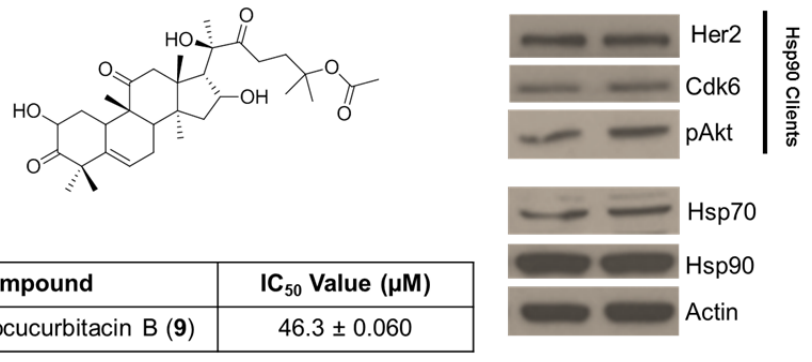
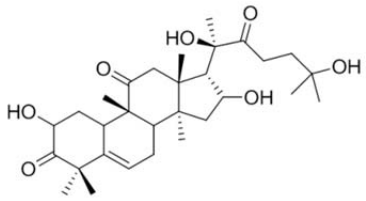


Figure 20 Western blot for Hsp90 client proteins (pAkt, Her2 and Cdk6) and heat shock proteins (Hsp70 and Hsp90) from cell lysates dosed for 24 hours with high and low concentrations (five times the IC_{50} value and half the IC_{50} value, respectively) of cucurbitacins that exhibit nanomolar IC_{50} values.



Compound	IC_{50} Value (μM)
23,24 Dihydrocucurbitacin B (**9**)	46.3 ± 0.060



Compound	MCF7 IC_{50} Value (μM)
23,24 Dihydrocucurbitacin D (**10**)	5.13 ± 0.006

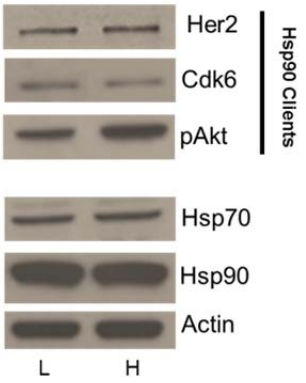


Figure 21 Western blot for Hsp90 client proteins (pAkt, Her2 and Cdk6) and heat shock proteins (Hsp70 and Hsp90) from cell lysates dosed for 24 hours with high and low concentrations (five times the IC_{50} value and half the IC_{50} value, respectively) of the 23,24-dihydrocucurbitacin B and 23,24-dihydrocucurbitacin D.

Incubation with high concentrations of **4** and **6** led to a decrease in client levels (Figure 19A), indicating disruption of client maturation. These cucurbitacins also decreased client levels in a dose-dependent manner at their IC_{50} values (Figure 19C). No changes in Hsp90 or Hsp70 levels were observed; however, Hsp27 levels were increased at high concentrations of **6**. The levels of Cdc37 and p23 remained constant at all concentrations (Figure 19A and B). Client and heat shock protein levels were unchanged after incubation with high and low concentrations of the remaining cucurbitacins (Figures 20 and 21). No client degradation at high concentrations of compound and constant heat shock protein levels at high and low concentrations indicate the

mechanism(s) by which these compounds inhibit MCF7 cancer cell growth is unrelated to Hsp90 inhibition.

III.4 Cucurbitacin D, but not 3-*epi*-isocucurbitacin D, induces client protein degradation through disruption of Hsp90-co-chaperone interactions

Concentration-dependent client degradation void of induction of the HSR indicates an alternative mechanism to traditional N-terminal inhibitors for preventing client maturation. Compounds such as **2** and **3** disrupt the Hsp90 heteroprotein complex and thereby prevent co-chaperone assistance during the chaperone cycle. In addition, concentrations of these compounds destabilize the heteroprotein-client complex and do not induce the HSR. There are several reports of **2** and **3** as Hsp90 modulators present in literature. These natural products disrupt interactions between Hsp90 and co-chaperones associated with both early and late stages of the chaperone cycle. Celastrol (**2**) was shown to covalently bind the Hsp90 N-terminus thereby disrupting Hsp90 from interacting with Cdc37, a co-chaperone involved during the early stages of the chaperone cycle.[54] Gedunin (**3**) binds the co-chaperone p23 and prevents p23 from facilitating the Hsp90 chaperone cycle.[31] Ultimately, these activities result in improper trafficking and localization of clients, such as the glucocorticoid receptor.

Consistent with previous studies, Figure 22A confirmed that high concentrations of **2** and **3** disrupted components of the heteroprotein complex, while the N-terminal inhibitor **1** did not disrupt these Hsp90-co-chaperone interactions. Co-immunoprecipitation of Cdc37 and p23 was disrupted in the presence of high concentrations of **2** and **3** when using an antibody that targets Hsp90. A high concentration of **4** also disrupted the Hsp90 heteroprotein complex (Figure 22B). Incubation with a high concentration of **4** completely disrupted Hsp90-Cdc37 and Hsp90-p23

interactions in MCF7 cell lysates. Interestingly, a high concentration of **6** had no effect on the interactions between Hsp90 and these co-chaperones; Hsp90-Cdc37 and Hsp90-p23 interactions remained intact at both high and low concentrations of **6** (Figure 22B).

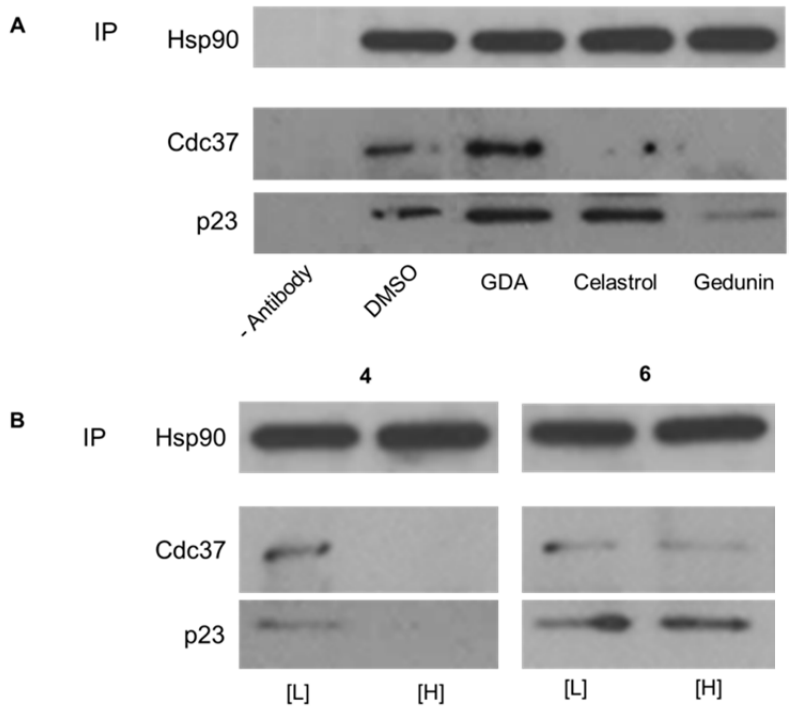


Figure 22 (A) Co-immunoprecipitation of Cdc37 and p23 using an anti-Hsp90 antibody (abcam) from MCF7 cell lysates treated for 24 hours with vehicle (0.25% DMSO) or high concentrations (5-times the IC_{50} value) of the controls geldanamycin (GDA; final concentration = 0.2135 μM) (**1**), celastrol (final concentration = 0.765 μM) (**2**), gedunin (final concentration = 54.5 μM) (**3**). (B) Co-immunoprecipitation of Cdc37 and p23 using an anti-Hsp90 antibody from cell lysates treated with high and low concentrations (5-times the IC_{50} value and half the IC_{50} value, respectively) of cucurbitacin D (final [H] concentration = 0.765 μM; final [L] concentration = 0.299 μM) (**4**) or 3-*epi*-isocucurbitacin D (final [H] concentration = 3.590 μM; final [L] concentration = 0.359 μM) (**6**).

These data suggested that, although **4** and **6** both result in client protein degradation, the mechanism by which these compounds disrupt client maturation are different. **4** disrupted

Hsp90-Cdc37 and Hsp90-p23 complexes, which ultimately led to a cellular decrease of client levels. **6** also reduced client levels, but did not affect Hsp90-co-chaperone interactions. This cucurbitacin may directly inhibit Hsp90 and bind an alternative site to the N-terminal ATP-binding pocket (e. g. C-terminal inhibitors, sansalvamide A, etc.), bind and inhibit the function of proteins important for client maturation (e. g. efrapeptins, cruentaren A, etc.) or disrupt interactions of other protein within the Hsp90 heteroprotein complex.[55-57]

III.5 High concentrations of cucurbitacin D do not increase cellular levels of Hsp27

Patwardhan et al. reported that **3** does not induce the HSR in several different cancer cell lines, including the cervical cancer cell line HeLa and the breast cancer cell lines Hs578T and MCF7.[31] More specifically, no increases in the levels of Hsp90, Hsp70 or Hsp27 were observed after incubation with concentrations up to 160 μM of **3**. To corroborate Western blot and co-immunoprecipitation data reported here and further verify that **4** disrupts client protein maturation in a manner comparable to **3**, Hsp27 levels were detected after incubation with high concentrations of **3**, **4** and **6**. Hsp27 levels were detected via Western blot analysis in Figure 23 (MCF7 cells incubated with 5- and 10-times the IC_{50} value of each compound). The levels of Hsp27 were unchanged after incubation with high concentrations of **3** and **4**, which were comparable to vehicle control; however, increased Hsp27 levels were observed at high concentrations of **6** (Figure 23 and Figure 19C).

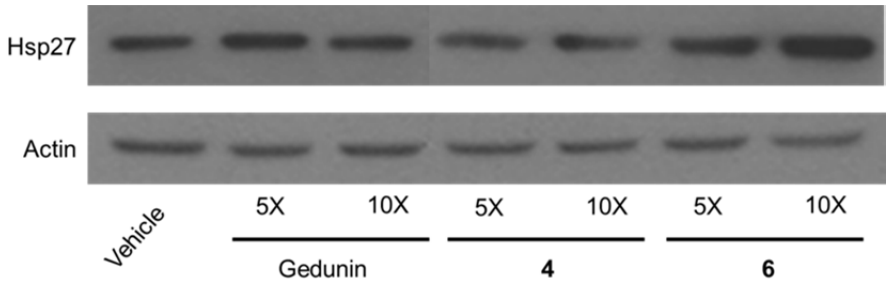


Figure 23 Western blot for Hsp27 and actin levels from MCF7 cell lysates treated for 24 hours with vehicle (0.25% DMSO), 5-times (5X) or 10-times (10X) the IC_{50} value of the gedunin (final 5X concentration = 54.5 μM; final 10X concentration = 109.0 μM) (**3**), cucurbitacin D (final 5X concentration = 0.765 μM; final 10X concentration = 5.980 μM) (**4**) and 3-*epi*-isocucurbitacin D (final 5X concentration = 3.590 μM; final 10X concentration = 7.180 μM) (**6**).

Collectively, these data suggest that **4** destabilized client maturation through similar mechanisms to **3** by disrupting Cdc37 and p23 from the Hsp90 heteroprotein complex; however, **4** exhibited a dramatic 18-fold increase in potency when compared to **3**. **6** also led to client degradation at nanomolar concentrations, but did not disrupt Hsp90-Cdc37 nor Hsp90-p23 interactions and appears to induce client degradation through an alternative mechanism. Like **3**, no increase in Hsp27 levels was observed at high concentrations of **4**, yet increased levels of Hsp27 were detected at high concentrations of **6**, further supporting an alternative mechanism for client degradation in the presence of these cucurbitacins. In conclusion, the curcurbitacins have been shown to affect multiple cellular pathways and exhibit potent inhibitory activity against many cancer cell lines. The data provided suggests the anti-proliferative activity manifested by cucurbitacin D and 3-*epi*-isocucurbitacin D is partly attributed to disruption of Hsp90 client protein maturation and cucurbitacin D destabilizes components of the Hsp90 heteroprotein complex, and ultimately results in client degradation.

III.6 Methods and Experimentals

General Procedures

Experimental data for each isolated compound was compared to previously reported literature data. NMR spectra were acquired on a Bruker AVANCE-400 MHz NMR spectrometer and compared to previously published data. High-resolution mass spectrometry (HRMS) data was obtained using either electron ionization (EI) on a ThermoFinnigan MAT 95 XL mass spectrometer or electrospray ionization (ESI) on a ThermoFinnigan LCQ Advantage Ion Trap liquid chromatography-mass spectrometer (LC/MS). Melting points were determined on a Vernier Melt Station melting point apparatus. TLC analysis was performed using pre-coated silica gel PE sheets (UV_{254}, 250 μm layer). Compounds were purified via column chromatography using silica gel 40-63 μm (230-400 mesh), preparative normal phase-TLC (silica gel, UV_{254}, 2 μm layer) and reverse phase HPLC using a Dynamax liquid chromatograph (Varian Chromatography Systems) with a PDA-2 photodiode array UV detector (Alltech preparative column, Econosil C18 10 μm, length 250 mm, ID 22 mm). Gradients of MeOH/H_2O or ACN/H_2O were utilized with a 9 or 13 mL/min flow rate. Analytical separations were optimized on an Alltima C18 (Alltech analytical column, 5 μm, length 250 mm, ID 4.6 mm) HPLC column using a flow rate of 1 mL/min then transferred to preparative scale, All reagents and solvents were obtained from commercial suppliers and used as received.

Isolation and Semi-syntheses of Cucurbitacins

Fruits from *Cucurbita texana* (Cucurbitaceae) were cut into pieces and homogenized with MeOH, filtered and the solvent was removed under reduced pressure. The residue was subjected to flash column chromatography with gradient elution (hexane/EtOAc and then EtOAc/MeOH of

increasing polarity). The fractions containing the same compounds as determined by TLC, were combined and subjected to further purification by one of three methods: normal phase column chromatography, preparative normal phase TLC, or preparative reverse phase HPLC[33-36]. The spectroscopic data for the pure isolated compounds were compared to published literature data: **1.** cucurbitacin D (**4**)[58-60], **2.** cucurbitacin B (**5**)[50, 61], **3.** 3-*epi*-isocucurbitacin D (**6**)[59, 60, 62]**, 4.** cucurbitacin I (**7**)[61, 63]**, 5.** cucurbitacin E (**8**)[61, 63]**, 6.** 23,24-dihydrocucurbitacin D (**9**)[64]**, 7.** 23,24-dihydrocucurbitacin B (**10**)[50, 65]**, 8.** data for multiple cucurbitacins[66-69].

Preparation of 3-*epi*-isocucurbitacin D (6). Cucurbitacin D (**4**) (50 mg, mmol) was added to a suspension of silica gel (3 g) in CH_2Cl_2 (5 mL). The reaction was heated to 40 ºC for 72 h. The solution was allowed to cool followed by dilution with EtOAc and filtration through plug of silica gel. The crude mixture was purified via preparative normal phase TLC to provide 3-*epi*-isocucurbitacin D (**6**) (13 mg) as a white solid in 25% yield.

Preparation of Cucurbitacin I (8). Palladium acetate (5 mg, 0.022 mmol, 0.3 equiv) was added to a vial containing cucurbitacin D (**4**) (44 mg, 0.085 mmol, 1 equiv), NaOAc (4 mg, 0.043 mmol, 0.5 equiv) and bromobenzene (0.01 mL, 0.095 mmol, 1.1 equiv) dissolved in dimethylacetamide (DMA) (0.40 mL). The vial was sealed with a teflon lined cap and the reaction was stirred at 80 ºC for 24 h. The reaction was allowed to cool and diluted with EtOAc (10 mL) and H_2O (5 mL), followed by extracting the aqueous layer three times with EtOAc. The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The crude product was initially purified via column chromatography using hexane/EtOAc to remove residual DMA. Minor impurities were further removed via preparative reverse phase HPLC

using MeOH/H_2O, resulting in the isolation of cucurbitacin I (**8**) (16 mg) as a white solid in 38% yield (unoptimized reaction conditions).

Preparation of 23,24-Dihydrocucurbitacin D (10) (Cucurbitacin R) and 23,24-Dihydrocucurbitacin B (11). 5% palladium on carbon (16 mg) was added to a 25 mL round bottom flask containing the desired cucurbitacin starting material (44.0 mg) dissolved in EtOH (5 mL). A septum was added to the flask, the headspace was flushed with H_2 and the reaction was stirred for 4 hours at 1 atm H_2. Upon completion, the reaction was filtered through a plug of silica gel using EtOAC. The crude product was purified using column chromatography (hexanes/EtOAc) to provide the desired dihydro compounds as white solids in 76–88% yield.

Antibodies and Reagents

Antibodies targeting Her2, Cdk6, phospho-Akt (pAkt), and Hsp27 were purchased from Cell Signaling Technology. Antibodies targeting Raf and actin were purchased from Santa Cruz Biotechnology. The remaining antibodies are listed and were purchased from the indicated vendors: Hsp90 (Enzo Life Sciences); Hsp70 (Assay Designs); Cdc37 and p23 (abcam). Gedunin was isolated in house. Celastrol was purchased from Cayman Chemical and geldanamycin was purchased from Sigma Aldrich.

Cell Culture

MCF7 cells were maintained in Advanced DMEM/F12 (1:1; Gibco) supplemented with streptomycin (500 µg/mL; corning cellgro), penicillin (100 units/mL; corning cellgro), L-glutamine (2 mM; corning) and 10% FBS (Atlanta Biologicals). Cells were grown in a humidified atmosphere (37 °C, 5% CO_2) and passaged when confluent.

Anti-proliferation

Cells were grown to confluence, seeded (2000 cells/well, 100 μL total media) in clear, flat-bottom 96-well plates and allowed to attach overnight. Varying concentrations of compound in DMSO (1% DMSO final concentration; Sigma-Aldrich) was added. Cells were returned to the incubator for an additional 72 h. After 72 h, cell growth was determined using an MTS/PMS cell proliferation kit (Promega) per the manufacturer's instructions. Cells that incubated in 1% DMSO were used as 100% proliferation (i.e. DMSO = 100% growth) and the relative growth for each compound concentration was compared to 1% DMSO. IC_{50} values were calculated from two separate experiments performed in triplicate using GraphPad Prism 6.0 (GraphPad Software).

Western Blot

MCF7 cells were grown to confluence and seeded at 0.4 x 10^6 cells/well/2 mL. Cells were incubated for 24 hours and treated with varying concentrations of compound in DMSO (0.25% DMSO final concentration), or vehicle (DMSO) for 24 hours. Cells were harvested in cold DPBS (corning cellgro) and lysed using MPER (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) according to the manufacturer's directions. Lysates were clarified at 14,000g for 10 minutes at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (20 μg) were suspended in Laemmli sample buffer (15 μL; BioRad) and boiled at 70^{o}C for 15 minutes. Samples were then electrophoresed under reducing conditions (10% and 12% acrylamide gels; made in house), transferred to PVDF (0.45 μm; Thermo Scientific), and immunoblotted with the corresponding antibody. Membranes were incubated with an appropriate horseradish peroxidase-

labeled secondary antibody (Santa Cruz Biotechnology and GE Healthcare), developed with a chemiluminescent substrate, and visualized.

Co-immunoprecipitation

MCF7 cells were grown to confluence and seeded at 2x106 cells/5 mL in 10 cm dishes. Cells were incubated for 24 hours and then treated with compound in DMSO (0.25% DMSO final concentration), or vehicle (DMSO) for 24 hours. Media and cells were collected with DPBS and centrifuged at 200g for 5 minutes at 4^{o}C. Supernatant was aspirated and pellets were washed one time with cold DPBS and centrifuged. Supernatant was aspirated and cell pellets were subsequently suspended in non-denaturing lysis buffer (20 mM Tris-HCl at pH 7.5; Fisher Scientific, 25 mM KCl; Sigma-Aldrich, 2 mM MgCl; Fisher Scientific, 2mM DTT; Sigma-Aldrich, 20 mM sodium molybdate; Sigma-Aldrich, 2mM ATP; Sigma-Aldrich and 0.1% NP-40; US Biological) supplemented with protease and phosphatase inhibitors (Roche) and incubated on ice for 2 hours. Lysates were clarified at 14,000g for 10 min at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal protein (400 µg) was incubated with 1 µg of Hsp90 antibody (H90.10; abcam) in 500 µL total volume lysis buffer for approximately 2 hours with rocking at 4 °C. Following incubation, 30 µL of re-suspended MagBeads Protein G (GenScript) was added to each sample and incubated with rocking for 1.5 hours at 4 °C. Protein G beads were washed 3 times with lysis buffer (500 µL), suspended in Laemmli sample buffer (15 µL) and boiled at 70^{o}C for 15 minutes to dissociate proteins from beads. Samples were electrophoresed under reducing conditions (12% acrylamide gels; made in house), transferred to PVDF, and immunoblotted with the indicated antibodies. Membranes were incubated with a species-

appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

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Chapter IV

Continued Optimization of Novobiocin-derived Hsp90 C-Terminal Inhibitors

IV.1 Targeting the Hsp90 C-Terminal Binding Pocket

Matts and co-workers discovered the Hsp90 C-terminus contains a second nucleotide-binding site. They observed that molybdate prevented geldanamycin from binding the Hsp90 N-terminus and that molybdate protected the C-terminus from proteolysis upon exposure to trypsin.[1] This observation was further supported when Neckers and co-workers determined that novobiocin, a coumarin-containing antibiotic, bound a carboxy-terminal Hsp90 fragment.[2] These researchers also demonstrated the C-terminal binding site of novobiocin also bound ATP. The amino acids 657-677 were crucial for maintaining novobiocin-C-terminal interactions and removal of these residues reduced Hsp90's affinity for novobiocin.[3] It was also discovered that novobiocin interacts with a C-terminal region of Hsp90 that is important for dimerization and co-chaperone binding.[3, 4] These data led to the hypothesis that inhibitors of the Hsp90 C-terminus may disrupt dimerization and ultimately prevent the chaperoning of client proteins. Agard and coworkers further examined the intra- and inter-Hsp90 monomer interactions and found that dimerization is required for ATP hydrolysis.[5] Incidentally, removal of the C-terminal dimerization domain resulted in reduced ATPase activity (approximately 6-10 fold), which further supported that disruption of C-terminal dimerization would prevent client maturation, as ATP hydrolysis is an integral part of the chaperone cycle.

Additional information about the location of the C-terminal binding site has been identified through the use of proteolytic fingerprinting and photoaffinity labeling.[6] It was shown that Hsp90 C-terminal inhibitors stall the Hsp90 complex in an open confirmation, which results in protection from proteolysis (via trypsin at Arg620 and Lys615). These findings were further

supported by Retzlaff and coworkers, who demonstrated that C-terminal inhibitors interact with and stabilize the C-terminal pivot point, which ultimately prohibits conformational changes required for chaperone activity.[6, 7]

IV.2 Novobiocin and Novobiocin-derived Analogs Exhibit Anticancer Activity Void of the Heat Shock Response

It was originally proposed that the coumermycin antibiotics, such as novobiocin (Figure 24), chlorobiocin, and coumermycin A1 could bind the Hsp90 N-terminal nucleotide-binding pocket due to complementary structural features within the binding site. Indeed, novobiocin was able to compete with the N-terminal inhibitors, radiciol and geldanamycin, for Hsp90 binding; however, neither geldanamycin nor radicicol could prevent novobiocin from binding Hsp90.[2, 8] To determine whether these inhibitors bound Hsp90 at different locations, amino- and carboxy-terminal fragments of Hsp90 were constructed, and as expected, geldanamycin bound the N-terminal fragment, but novobiocin was shown to interact with only the C-terminal region.[2]

Although novobiocin was found to bind the Hsp90 C-terminus, it manifested low anti-proliferative activity in cancer cell models (~700 μM in SkBr3). To improve activity, structure-activity relationship (SAR) studies have been pursued. The first reported novobiocin analog was compound A4 (Figure 24), in which the benzamide side chain was replaced with an acetamide moiety and the coumarin ring lacked the 4-hydroxyl and the 3'-carbamate.[9] A4 induced client protein degradation at approximately 10 μM in the LNCaP prostate cancer cell line, which is a mutated androgen receptor-dependent prostate cancer cell line.[9] Although A4 was able to segregate the HSR from client protein degradation, A4 robustly induced the HSR at concentrations that were significantly lower than the concentrations needed for client degradation.[9] The ability of A4 to cause increased levels of chaperones without client

degradation was subsequently utilized in models of protein mis-folding diseases and led to the evaluation of A4 as a neuroprotective agent.[9]

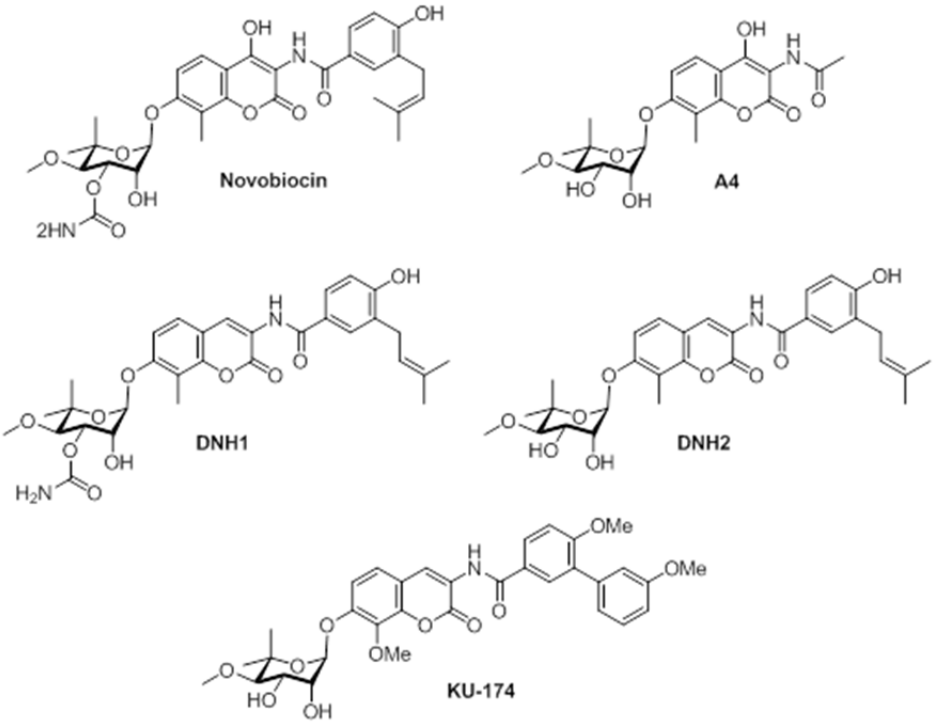


Figure 24 Inhibitors of the Hsp90 C-terminus.[10]

4-Deshydroxynovobiocin (DHN1) and 3'-descarbamoyl-4-deshydroxynovobiocin (DHN2) (Figure 24), were synthesized to evaluate the importance of the 4-hydroxy to Hsp90 inhibitory activity.[11] Removal of the 4-hydroxy decreased activity against DNA gyrase, and improved Hsp90 inhibitory activity.[11] Furthermore, client protein degradation was observed at concentrations of DHN1 and DHN2 that did not increase heat shock protein levels. Further modifications to the coumarin core and amide side chain led to the synthesis of the most potent compound, KU-174 (Figure 24). KU-174 was subjected to a NCI60 screen and showed activity against multiple cancer cell lines.[12] Other investigational studies on the coumarin core include

compound **2** (Figure 25) as well as replacement of the coumarin core with a biphenyl moiety (Figure 25; compound **3**).[13-17] Analogs containing the biphenyl ring system manifest potent anti-proliferative activity against both SKBr3 and MCF-7 cell lines and represent a new paradigm for Hsp90 C-terminal inhibition.[18]

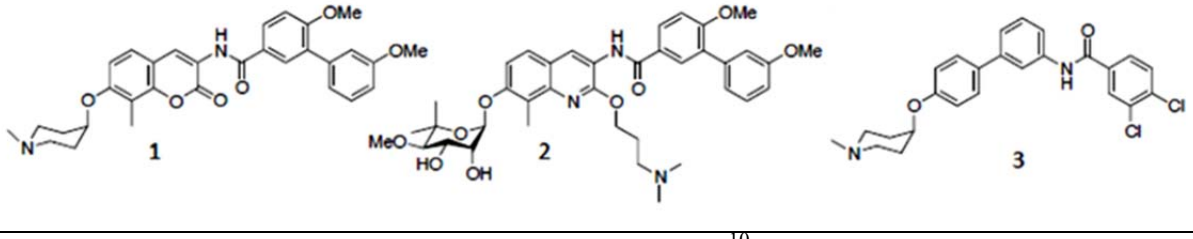


Figure 25 Additional inhibitors of the Hsp90 C-terminus.[10]

IV.3 Structure-activity relationship studies of novobiocin's amide linker

Increasing evidence suggests that the incorporation of hydrogen bond donors on the amide linker and side chain are beneficial.[19] Analogs containing a 2- or 3-indole, or, a 4-methoxyphenyl side chain exhibit anti-proliferative activity against several cancer cell lines as well as Hsp90 inhibitory activity via client protein degradation.[19-21] Therefore, it was proposed that incorporation of a hydrogen bonding network within the linker of analogs containing the biphenyl replacement could further increase inhibitory activity. Indeed, analogs that contained a triazole in lieu of the amide linker and biphenyl replacements of the coumarin core exhibited potent anti-proliferative activity against several cancer cell lines (Table 7).[22]

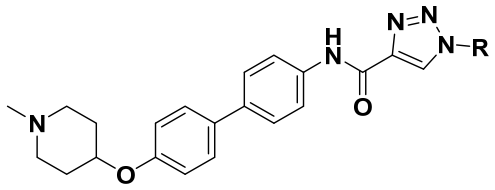


Table 6 Anti-proliferative activity of selected biphenyl triazole analogs.						
R	**SKBr3**	**MCF-7**	**MDA-1986**	**JMAR**	**PC3-MM2**	**LNCaP**
Bn (**4**)	0.17±0.02	0.50±0.02	0.28±0.10	0.50±0.06	0.55±0.46	0.65±0.54
4-MeBn (**5**)	0.17±0.03	0.12±0.01	0.36±0.05	0.42±0.07	0.36±0.04	0.20±0.04
4-MeOBn (**6**)	0.16±0.02	0.27±0.06	0.48±0.09	0.61±0.09	0.44±0.19	0.24±0.07

For these analogs, decreased levels of client proteins and no increased heat shock protein levels were observed, indicating Hsp90 C-terminal inhibition (Figure 26A and B).

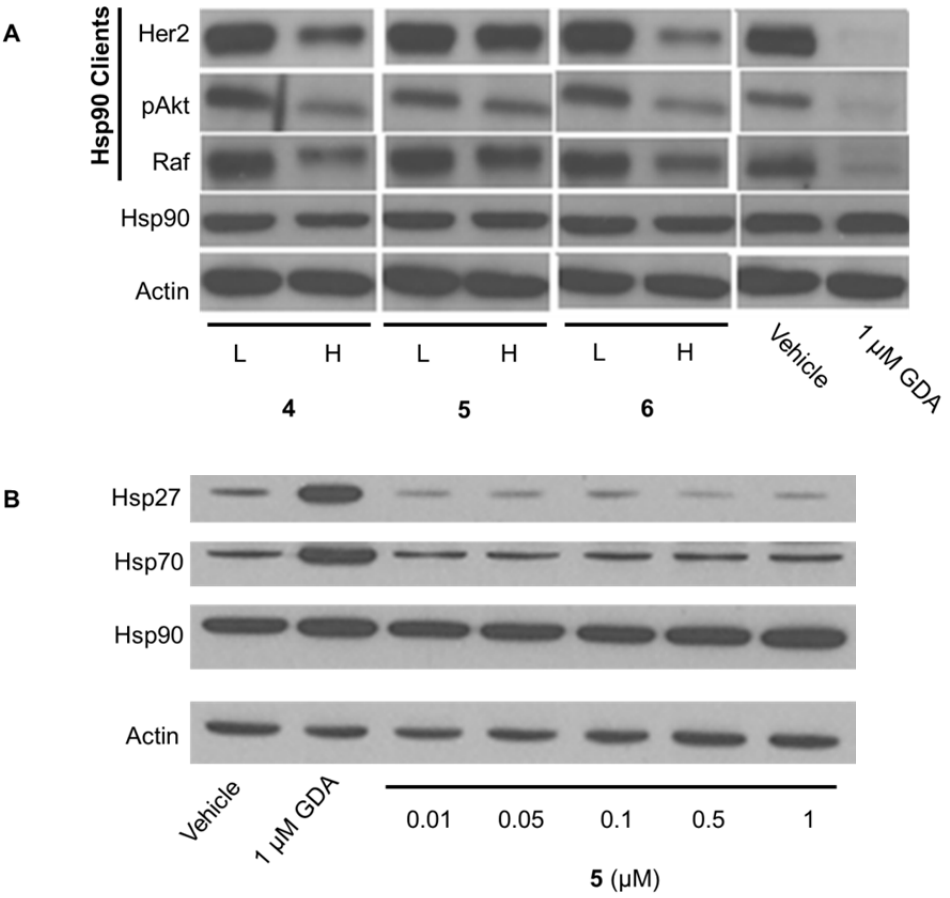


Figure 26 (A) Western blot for Hsp90 client proteins (pAkt, Her2 and Cdk6) and Hsp90 from cell lysates dosed for 24 hours with 1 μM geldanamycin, vehicle (1% DMSO) or high and low concentrations (five times the IC_{50} value and half the IC_{50} value, respectively) of **4**, **5** or **6**, (B) Western blot for heat shock protein levels (Hsp27, Hsp70 and Hsp90) in the presence of 1 μM geldanamycin, vehicle or increasing concentrations of **5**.

Proteolytic fingerprints were generated in the presence of high concentrations of **5** (Table 6) and novobiocin. As shown in Figure 27, Western blot for C-terminal fragments of Hsp90 after proteolytic cleavage detected the emergence of a 50 kDa band in the presence of high concentrations of **5** and novobiocin that was absent in vehicle control. Together, these

observations highlight the significance of hydrogen bonding interactions at this location of the Hsp90 C-terminal binding pocket.

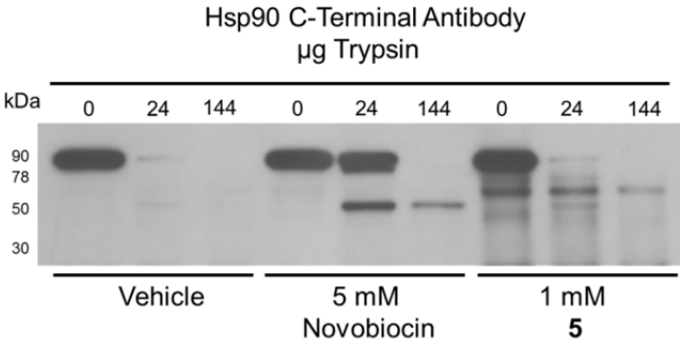


Figure 27 Proteolysis of Hsp90 from TnT reticulocyte lysate incubated under conditions of protein synthesis with vehicle (1% DMSO), 5mM novobiocin, and 1mM **5**. An antibody specific to the C-terminus of Hsp90 was used to identify the Hsp90 fragments produced in the presence of increasing amounts of trypsin.

In addition to the coumarin core and benzamide side chain, novobiocin contains a noviose sugar. Extensive SAR studies have been carried out on noviose.[23-26] It was determined that the 2'- and 3'-hydroxy moieties are crucial for maintaining activity, whereas, the 6'-gem-dimethyl group and the 5'-methoxy are expendable.[23] Additional studies demonstrated that N-methyl piperidine is a suitable replacement for the noviose sugar (compound **1** in Figure 28).

IV.4 The Transformation of Hsp90 Inhibitors Into MAPK Inhibitors

Additional studies on novobiocin's amide linker led to the development of analogs that contain a urea surrogate. Similar to the triazole, it was hypothesized that the urea moiety could contribute a hydrogen bonding network and generate analogs that exhibit improved Hsp90 inhibitory activity. Indeed, many urea-containing analogs exhibited potent anti-proliferative activity against several cancer cell lines. However, some of the compounds did not inhibit Hsp90. Investigation of these analogues led to the identification of **8**, a novobiocin analogue that

exhibited potent anti-proliferative activity against the MCF7 breast and A549 lung cancer cell lines without Hsp90 inhibitory activity. Instead, compound **8** was shown to disrupt mitogen-activated protein kinase (MAPK) signaling in a time-dependent manner, and to inhibit the phosphorylation of MEK and ERK. Since oncogenic mutations frequently occur within the MAPK pathway, much research has been devoted to the development of inhibitors of this pathway, including EGFR, Raf, MEK and ERK.[27-29] Preliminary SAR studies of **8** examined the necessity of each methoxy substitution on the biaryl side chain and identified moieties that transitioned novobiocin analogues from Hsp90 inhibitory activity, such as compound **7**, to MAPK inhibitors (Figure 28).

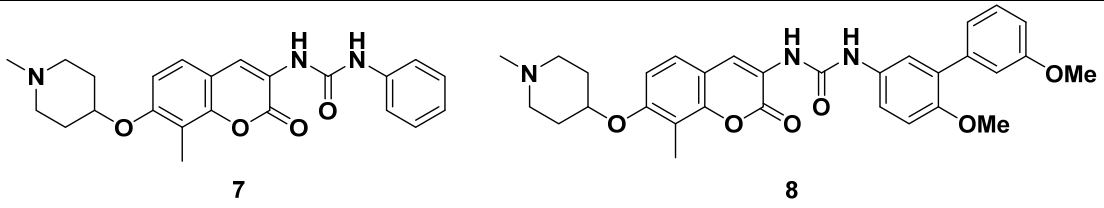


Figure 28 Structures of compounds **7** and **8**.

As previously mentioned, many client proteins that depend upon Hsp90 are up-regulated and represent signaling pathways that have been hijacked during malignant transformation.[30-33] Compounds that inhibit Hsp90, or the Hsp90 chaperone cycle, lead to client protein degradation via the ubiquitin proteasome pathway and deprive cancer of clients that drive progression and growth.[34-37] Therefore, decreased client levels are observed in a dose-dependent manner at the IC_{50} values obtained in cell proliferation assays for Hsp90 inhibitors. To determine whether a compound inhibits Hsp90, IC_{50} values are generated against cancer cell lines that depend upon functional Hsp90. The estrogen receptor-expressing MCF7 breast cancer cell line and the epidermal growth factor receptor-driven A549 lung cancer cell line both depend upon Hsp90 for

client protein maturation. Therefore, compounds that exhibit potent anti-proliferative activity against these cell lines may inhibit Hsp90. Table 7 shows the IC_{50} values for compounds **7** and **8** generated against these cancer cell lines, as well as the normalized cell line, MRC-5. **7** and **8** were shown to exhibit potent inhibitory activity of cancer cell growth compared to the MRC-5 line, indicating the cellular target(s) for these compounds facilitate cancer growth.

Table 7 IC_{50} values determined for compounds 7 and **8** against the MCF7 and A549 cancer cell lines as well as the normal human cell line, MRC5.

	IC_{50} Values (μM)	
Cell Line	**7**	**8**
MCF7	0.77 ± 0.01	0.17 ± 0.07
A549	0.24 ± 0.00	0.15 ± 0.02
MRC-5	3.6	4.3

Cellular protein client levels in MCF7 and A549 cells were determined via Western blot analysis after incubation with low and high concentrations of **7** and **8** (L = half the IC_{50} value; H = five-times the IC_{50} value, respectively; Figure 29). If client levels are unaffected upon incubation with low concentrations of the compound and decreased at high concentrations, the compound is likely to inhibit Hsp90 function.

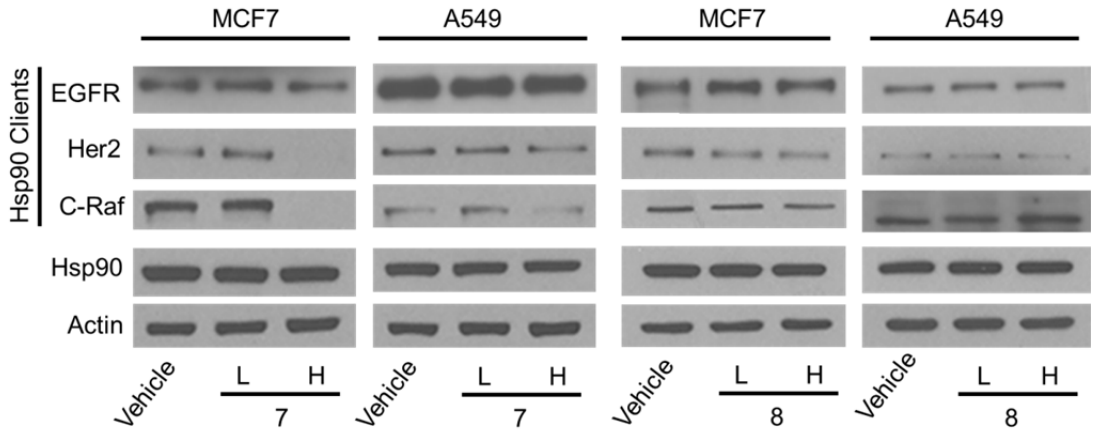


Figure 29 Western blot for Hsp90 client proteins (EGFR, Her2 and Raf) and Hsp90 using MCF7 and A549 cell lysates treated for 12 hours with vehicle (0.25% DMSO), a low concentration (L; half the IC_{50} value) or a high concentration (H; five-times the IC_{50} value) of compound **7** or **8**.

Incubation with high concentrations of **7** led to decreased levels of the clients EGFR, Her2 and C-Raf in both MCF7 and A549 cell lysates, when compared to vehicle control (Figure 30). Hsp90 levels at low and high concentrations of **7** were comparable to vehicle and indicative of targeting the Hsp90 C-terminus.[38] Furthermore, incubation with the N-terminal inhibitor, geldanamycin (GDA), led to decreased client levels at concentrations that increased cellular levels of Hsp90 (Figure 30).[39, 40] These data suggest the mechanism of action manifested by compound **7** is inhibition of the Hsp90 C-terminus, as opposed to the N-terminus.

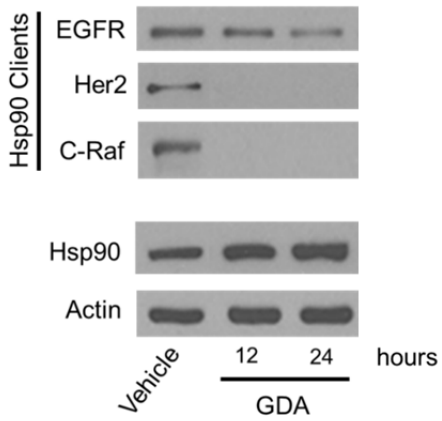


Figure 30 Western blot for Hsp90 client proteins (EGFR, Her2 and C-Raf) and Hsp90 from A549 cell lysates dosed for 12 and 24 hours with a high concentration (five times the IC_{50} value) of geldanamycin (GDA) or vehicle (0.25% DMSO).

In contrast, client protein levels were unchanged after incubation with low and high concentrations of **8** against both MCF7 and A549 cells. Since no Hsp90-dependent client protein degradation was observed with compound **8**, the data suggests this compound inhibits cancer cell growth unrelated to Hsp90 inhibition. Given the potent anti-proliferative activity manifested by compound **8** against both cancer cell lines as well as the selective inhibition of cancer cell growth, the mechanism of action for **8** was pursued.

Since **2** exhibited the most potent anti-proliferative activity against the non-small cell lung cancer cell line A549 and generated an IC_{50} value of 0.15 ± 0.02 μM, subsequent studies were initiated with this cell line. The A549 cell line expresses wild type B-Raf, C-Raf and mutant KRAS. Proliferation of the A549 cell line is driven by over-activation/expression of proteins involved in the Ras-Raf-MEK-ERK (MAPK) pathway and consequently, this cell line is commonly utilized to identify small molecule kinase inhibitors that target Raf, MEK and/or

ERK.[41] Interestingly, many inhibitors that target kinases involved in the MAPK pathway display structural similarities to compound **8** (Figure 31).

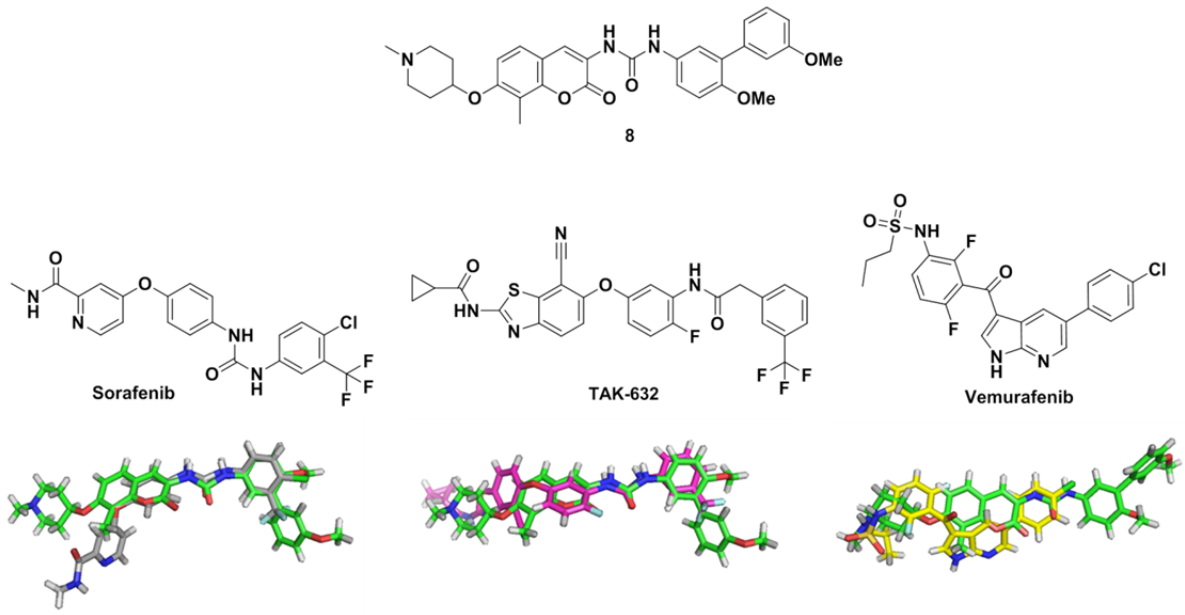


Figure 31 Structures and the overlay of compound **8** (green) with sorafenib (gray; a kinase inhibitor that targets B-Raf and C-Raf), with TAK-632 (magenta; a kinase inhibitor that targets C-Raf and wild-type B-Raf) and with vemurafenib (yellow; an inhibitor of mutant B-Raf).

Given the potent anti-proliferative activity exhibited by **8** against the MAPK-driven A549 cancer cell line, and the structural similarities between **8** and known disruptors of this pathway, it was hypothesized that **8** may inhibit the A549 cancer growth through disruption of MAPK signaling. Therefore, to determine whether **8** inhibits the kinases within the MAPK pathway, Western blots for phosphorylated proteins involved in the MAPK pathway were performed using A549 lysates treated with **8** at five-times the IC_{50} value (750 nM) for two to 12 hours (Figure 33B). Treatment with **8** led to a time-dependent decrease in phosphorylated MEK and ERK suggesting this compound inhibits kinase activity; however, decreased levels of phospho-MEK

(p-MEK) and phospho-ERK (p-ERK) were observed at different incubation times. Decreased p-MEK levels occurred after four hours of incubation with **8** and were undetectable after 12 hours of incubation. In contrast, decreased p-ERK levels were only observed after six hours of incubation and continued to decrease during the 12 hour incubation. These data indicate that **8** disrupts kinase activity upstream of MEK and ERK; **8** prevented phosphorylation of MEK, which subsequently prevented phosphorylation of ERK. The levels of other proteins crucial for MAPK signaling, such as EGFR and C-Raf, remained constant at each time point and were comparable to vehicle control. C-Raf is an Hsp90 client and undergoes proteolytic degradation upon 12 hours of incubation with Hsp90 inhibitors. After 12 hours of incubation with **8**, C-Raf levels were similar to vehicle and remained constant at each time point, which demonstrates that compound **8** inhibits cancer cell growth by a mechanism unrelated to Hsp90 inhibition. Total MEK and ERK levels also remained constant at each time point and were comparable to vehicle, indicating that decreased p-MEK and p-ERK levels were not the result of decreased MEK and ERK levels. To determine whether **8** inhibits general kinase activity, Western blots were generated for phosphorylated Akt (p-Akt) and total Akt levels using A549 cell lysates after two to 12 hours of incubation. Akt is phosphorylated by phosphoinositide 3-kinase (PI3K), and p-Akt formation is unrelated to the MAPK pathway.[42] Figure 32 demonstrates that p-Akt and total Akt levels were unaffected at each time point, which strongly suggests that **8** preferentially inhibits the phosphorylation of MAPK pathway proteins.

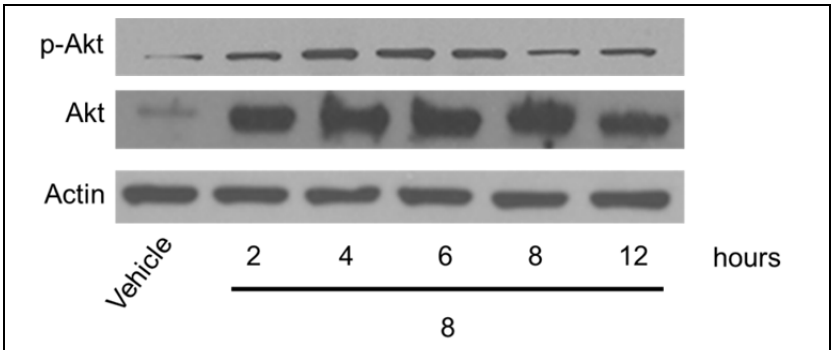


Figure 32 Western blot for Akt and p-Akt from A549 cell lysates dosed for the indicated amount of time with five times the IC_{50} value of compound **8** or vehicle (0.25% DMSO).

Figure 33A shows a concentration-dependent decrease in p-MEK and p-ERK after 12 hours of incubation with **8**. A low concentration of **8** (L; half the IC_{50} value) had little effect on cellular levels of p-MEK and p-ERK; however, incubation with a high concentration of **8** (five-times the IC_{50} value) resulted in dramatically decreased levels of p-MEK and p-ERK and was consistent with previous observations. Little effect was observed on the levels of other MAPK-associated proteins, as well as total MEK and ERK levels at either concentration.

It is well established that small molecule inhibition of the MAPK pathway activates multiple feed-back mechanisms that ultimately result in up-regulated MAPK pathway activity.[43-46] *In vitro*, this occurs at short incubation times and can be observed up to 24 hours after incubation with inhibitors of the MAPK pathway (via increased levels of p-MEK and p-ERK as well as increased transcription of MAPK proteins and proteins that support feed-back mechanisms). Western blot analysis of MAPK pathway proteins, p-MEK and p-ERK from A549 cell lysates treated with a high concentrations of **8** (750 nM) for 12 and 24 hours were conducted (Figure 33B). After 24 hours of incubation, p-MEK and p-ERK levels were only slightly increased compared to 12 hours of incubation; however, these levels remained lower than vehicle

control, which could result from the up-regulation of MAPK pathway activity via a feed-back mechanism or the consequence of chemical instability for compound **8**, which produces metabolites that no longer interact with the cellular target(s) within the MAPK pathway.

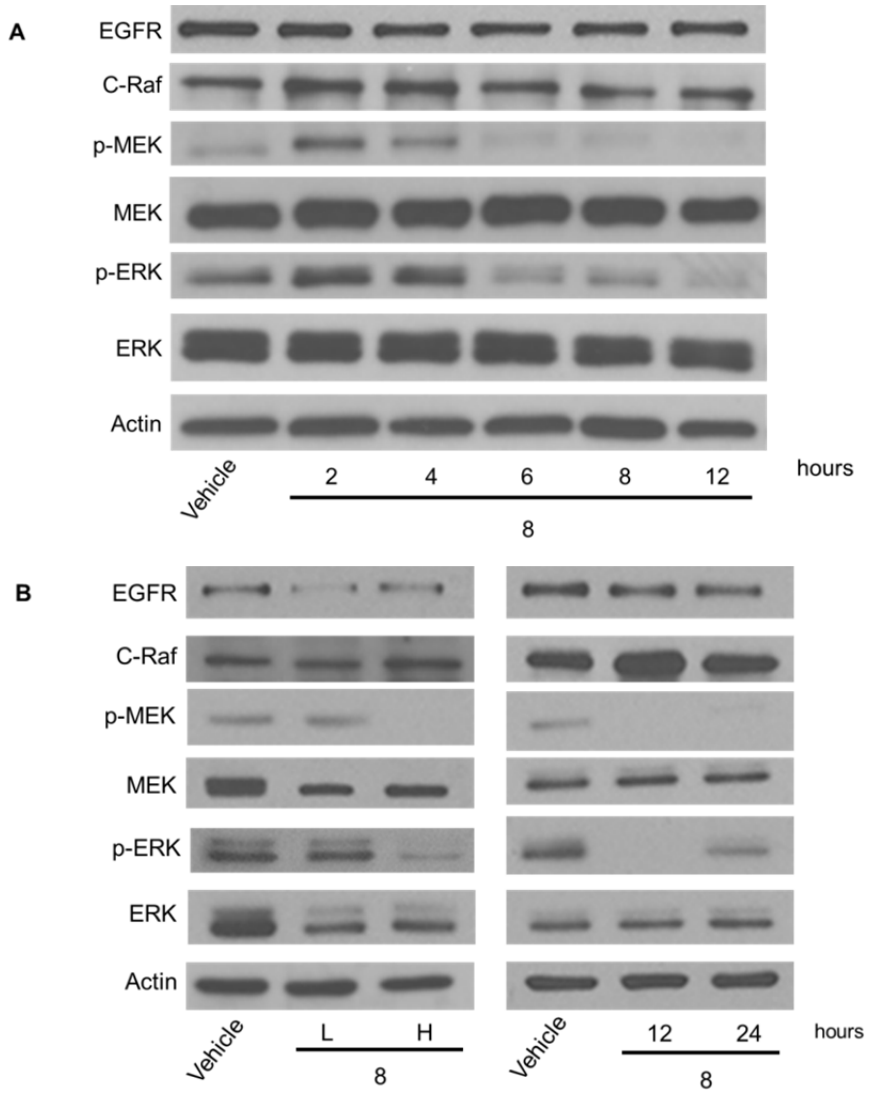


Figure 33 (A) Western blot for proteins of the MAPK pathway (EGFR, C-Raf, MEK, ERK) and phosphorylated MEK and ERK (p-MEK and p-ERK) from A549 cell lysates treated for two to 12 hours with five times the IC_{50} value (H) of **8** or vehicle (0.25% DMSO) (B) Western blot for proteins of the MAPK pathway and phosphorylated MEK and ERK from A549 cell lysates treated with vehicle (0.25% DMSO), 12 hours with half the IC_{50} value (L) and five-times the IC_{50} value (H) of **8** and 12 and 24 hours with five-times the IC_{50} value (H) of **8**.

IV.5 The novobiovin-derived MAPK inhibitor, 8, does not activate MAPK pathway feed-back mechanisms

To determine whether re-establishment of detectable p-MEK and p-ERK levels after 24 hours of incubation with **8** was the result of feed-back mechanism activation, Western blotting for Her3 and wild-type B-Raf levels were performed. Her3 and wild type B-Raf are expressed at relatively low levels in cancer cells under normal conditions. However, inhibition of MAPK pathway activity via small molecule EGFR, Raf or MEK inhibitors induce the increased expression of Her3 and wild-type B-Raf to compensate for decreased p-MEK and p-ERK signaling. After 24 hours of incubation with **8**, Her3 and wild-type B-Raf levels were comparable to vehicle and indicated no activation of a feed-back mechanism (Figure 35). Western blots for Her3 and wild-type B-Raf levels from A549 lysates treated with control compounds (sorafenib, vemurafenib and TAK-632) for 12 and 24 hours were also performed (Figure 34).

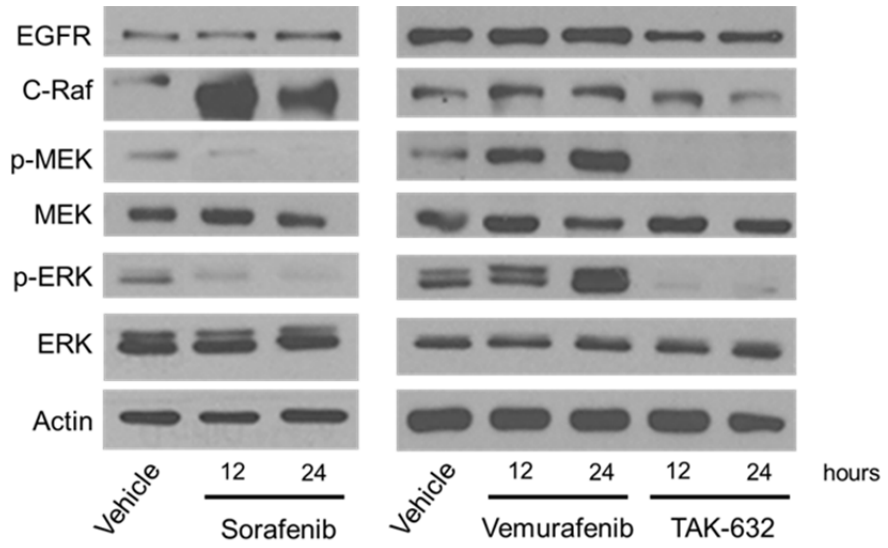


Figure 34 Western blot for phosphorylated and non-phosphorylated proteins of the MAPK pathway from A549 cell lysates dosed for 12 or 24 hours with five times the IC_{50} value of sorafenib (kinase inhibitor), vemurafenib (mutant B-Raf inhibitor), TAK-632 (wild type B-Raf and C-Raf inhibitor) or vehicle (0.25% DMSO).

Incubation with sorafenib at five-times the reported IC_{50} value (14 μM) resulted in decreased p-MEK and p-ERK levels and a time-dependent increase in Her3 levels (Figure 34 and 36).[41] Vemurafenib and TAK-632 are Raf inhibitors. Vemurafenib is a mutant B-Raf inhibitor that is relatively inactive against the A549 cell line, which expresses only wild-type B-Raf. Incubation with a high concentration of vemurafenib (five-times the reported IC_{50} value against the A549 cancer cell line, >50 μM) had little effect on Her3 and wild-type B-Raf levels, as well as MAPK pathway signaling after 12 and 24 hours (Figure 34 and 36).[47] TAK-632 is a kinase inhibitor that preferentially inhibits wild-type B-Raf and C-Raf, and ultimately leads to decreased p-MEK and p-ERK levels (Figure 34). Her3 and wild-type B-Raf levels remained unaffected after 12 and 24 hours of incubation with TAK-632 at five-times the reported IC_{50} value (8.5 μM) which is comparable to **8** (Figure 35).[48] These data indicate that **8** prevents p-

MEK and p-ERK formation via a manner similar to TAK-632 and targets wild-type B-Raf and C-Raf.

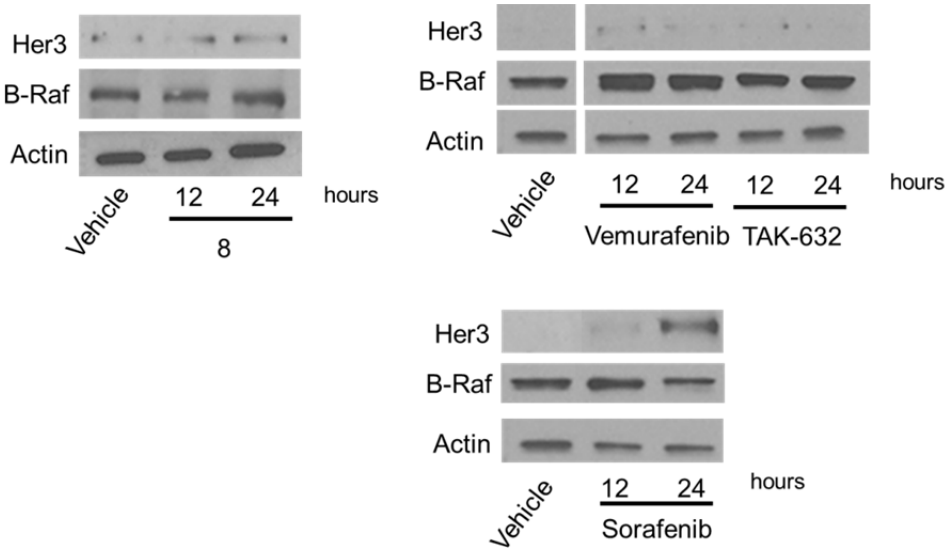
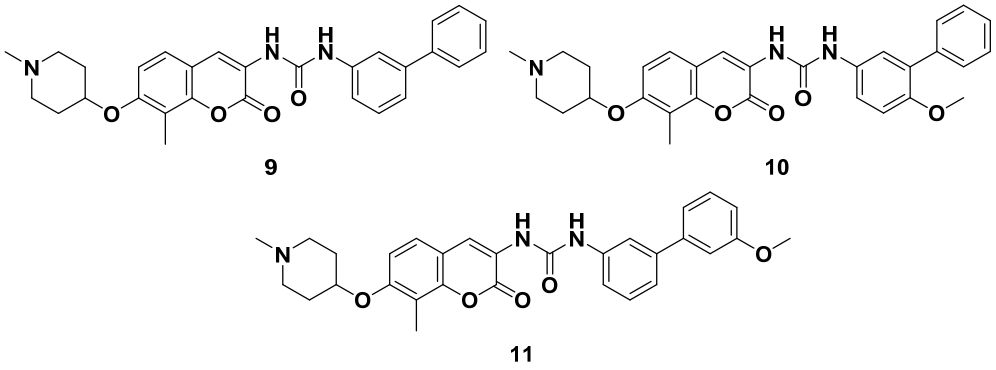


Figure 35 Western blot for proteins Her3 and wild-type B-Raf from A549 cell lysates treated for 12 or 24 hours with five-times the IC_{50} value of **8**, sorafenib, vemurafenib, TAK-632 or vehicle (0.25% DMSO).

IV.6 Preliminary structure-activity relationships designate Hsp90 inhibition and MAPK pathway disruption

Incorporation of a phenyl ring onto the urea linker (**7**) results in Hsp90 inhibition as observed via client protein degradation, while replacement of the phenyl substituent with a bulky di-methoxy, biaryl side chain leads to disruption of MAPK pathway activity. Therefore, compounds **9**, **10** and **11** contain substitutions about the biaryl side chain were synthesized and utilized to elucidate the substitutions that distinguish Hsp90 inhibitory activity from disruption of the MAPK pathway. **9** contains a biaryl side chain with no methoxy groups, while **10** and **11** each contain a single methoxy substitution on the biaryl side chain. IC_{50} values were determined

against the A549 cancer cell line and are reported in Table 8. Each compound exhibited potent anti-proliferative activity within the nanomolar range.



9 **10** **11**

Table 8 IC_{50} values of compounds **9**, **10** and **11** against the A549 lung cancer cell line.

Compound	**A549 Cell Line IC_{50} Values (μM)**
9	0.06 ± 0.02
10	0.41 ± 0.13
11	0.28 ± 0.04

Western blot analyses of Hsp90 clients were performed using A549 cell lysates dosed for 12 and 24 hours with a high concentration of each compound (five-times the IC_{50} value) to identify Hsp90 inhibitors (Figure 36A). After 24 hours of incubation with **9**, decreased levels of Hsp90-dependent client proteins were observed and constant levels of Hsp90, which suggests **9** is likely an Hsp90 C-terminal inhibitor. Mono-methoxy substitutions on the biaryl side chain (**10** and **11**) did not result in decreased client levels after 12 and 24 hours of incubation, indicating no Hsp90 inhibition. Western blot analyses for MAPK pathway proteins and p-MEK and p-ERK levels were performed using the A549 cell lysates dosed for 12 and 24 hours with **9** and **10** and are shown in Figure 36B. 12 hours of incubation with **10** led to decreased p-MEK and p-ERK

levels compared to vehicle; however, p-MEK and p-ERK levels were re-established after 24 hours of incubation. P-ERK levels were decreased after 12 hours of incubation with **11**; however, 24 hours of incubation resulted in p-ERK levels that were comparable to vehicle. After 12 and 24 hours of incubation with **11**, p-MEK levels were unchanged.

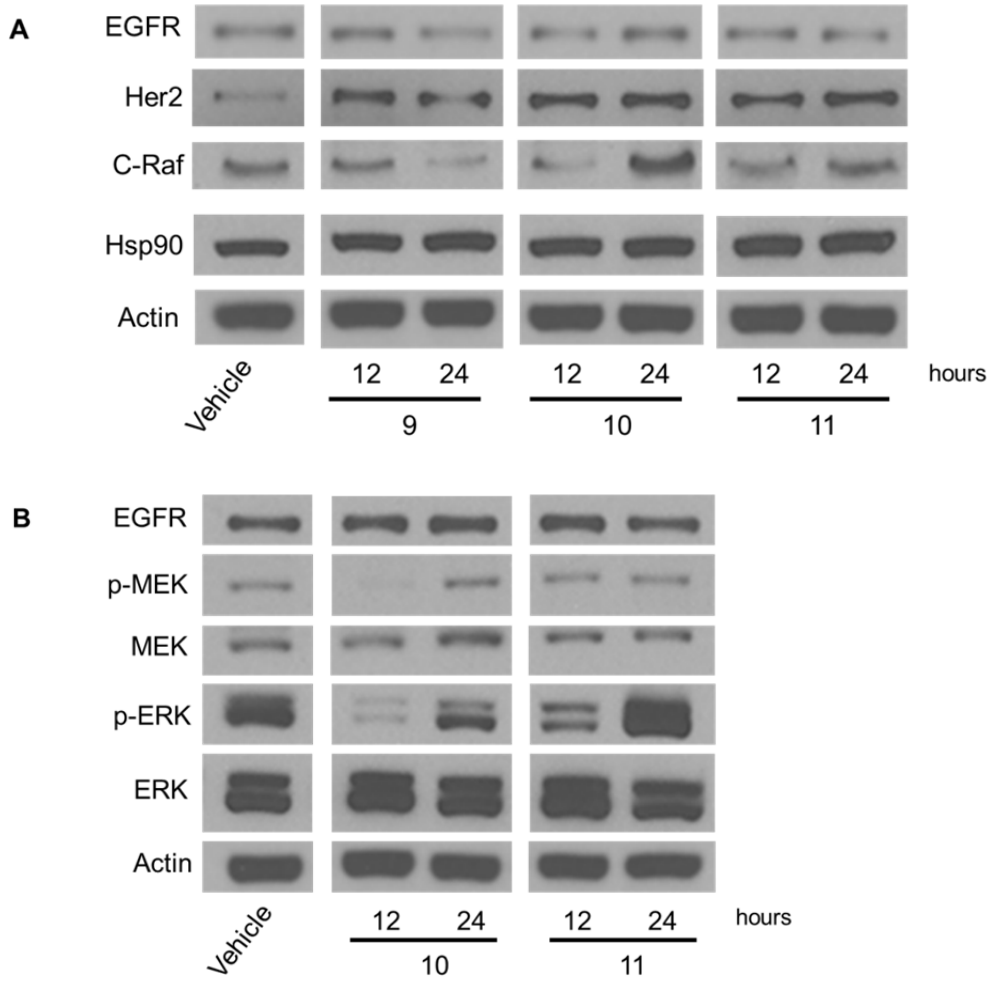


Figure 36 (A) Western blot for Hsp90 client proteins (EGFR, Her2 and C-Raf) and Hsp90 using A549 cell lysates treated for 12 or 24 hours with a high concentration (five-times the IC_{50} value) of **9**, **10**, **11** or vehicle (0.25% DMSO). (B) Western blot for proteins of the MAPK pathway (EGFR, C-Raf, MEK, ERK) and phosphorylated MEK and ERK (p-MEK and p-ERK) from A549 cell lysates treated for 12 or 24 hours with five-times the IC_{50} value of **10**, **11** or vehicle (0.25% DMSO).

These data indicate that both methoxy substitutions on the biaryl side chain are important for prolonged MAPK pathway disruption. While mono-methoxy substitutions inhibited p-MEK and/or p-ERK formation, neither substitution alone was able to sustain inhibitory activity to the extent of the dimethoxy-substituted side chain present in **8**.

While **8** was synthesized during SAR studies of potential novobiocin-based Hsp90 C-terminal inhibitors that contain urea surrogates of the amide linker, **8** failed to exhibit Hsp90 inhibitory activity and upon further investigation, **8** was shown to disrupt the MAPK pathway signaling. After 4 hours of incubation, **8** prevented p-MEK formation, which ultimately disrupted p-ERK formation. Disruption of p-MEK and p-ERK formation was comparable to the control compound, TAK-632; however, **8** exerted this activity at one tenth the IC_{50} value of TAK-632 (IC_{50} value of 0.15 ± 0.02 μM versus 1.7 μM against the A549 lung cancer cell line). While individual methoxy substitutions on the biaryl side chain tentatively inhibit p-MEK and/or p-ERK formation, the dimethoxy-substituted biaryl sidechain is required for sustained activity. Therefore, **8** represents a novel, novobiocin-based scaffold for the development of MAPK pathway inhibitors and appears to manifest a similar mechanism of action to TAK-632, which targets C-Raf and wild-type B-Raf.

IV.8 Methods and Experimentals

Antibodies and Reagents

Antibodies targeting Akt 1/2/3 and actin were purchased from Santa Cruz Biotechnology. The antibody targeting Hsp90 was purchased from Thermo Scientific (PA3-013). The following antibodies were purchased from Cell Signaling: EGF Receptor, p44/42 MAPK, P-p44/42 MAPK, MEK1/2, P-MEK1/2, pAkt, BRaf, CRaf, Her3/ErbB3 and Her2/ErbB2. Geldanamycin was purchased from Sigma Aldrich and sorafinib, vemurafinib and TAK-632 were purchased from Selleckchem.

Cell Culture

The media for each cell line was supplemented with streptomycin (500 μg/mL), penicillin (100 units/mL), and 10% FBS. MCF7 cells were maintained in Advanced DMEM/F12 (1:1; Gibco) supplemented with L-glutamine (2 mM). A549 cells were maintained in F12K (Cellgro). MRC-5 cells were maintained in DMEM (Cellgro). Cells were grown in a humidified atmosphere (37 °C, 5% CO2) and passaged when confluent.

Anti-proliferation

Cells were grown to confluence, seeded (2000 cells/well, 100 μL total media) in clear, flat-bottom 96-well plates and allowed to attach overnight. Compound at varying concentrations in DMSO (1% DMSO final concentration) was added. Cells were returned to the incubator for an additional 72 h. After 72 h, cell growth was determined using an MTS/PMS cell proliferation kit (Promega) per the manufacturer's instructions. Cells that incubated in 1% DMSO were used as 100% proliferation (i.e. DMSO = 100% growth) and the relative growth for each compound concentration was compared to 1% DMSO. IC50 values were calculated from two separate experiments performed in triplicate using GraphPad Prism 6.0.

Western Blot

Cells were grown to confluence and seeded at 0.4 x 106 cells/well/2 mL. Cells were incubated for 24 hours and treated with compound in DMSO (0.25% DMSO final concentration), or vehicle (DMSO) for the indicated amount of time. Cells were harvested in cold PBS and lysed using MPER (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) according to manufacturer's directions. Lysates were clarified at 14,000g for 15 minutes at 4° C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (15 μg) were electrophoresed under

reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with the corresponding antibody. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Proteolytic Fingerprinting Assay

Rabbit reticulocyte (Green Hectares) incubated under conditions of protein synthesis at 30oC in the presence of compound or vehicle (1% DMSO) for 10 minutes. Each reaction mixture contained 66.6% rabbit reticulocyte and 33.3% ATP regenerating system (10 mM creatine phosphate and 20 µg/mL creatine phosphokinase) and a final concentration of 75 mM KCl. Each reaction mixture contained the indicated amount of compound. After incubating, the samples were immediately placed on ice and the indicated amount of TPCK-treated trypsin (Worthington) was added to each sample. The samples digested on ice for an additional 6 minutes and the reactions were quenched by the addition of Laemmli sample buffer followed by immediate boiling. Equal amounts of each sample were electrophoresed under reducing conditions (10% acrylamide gels), transferred to PVDF, and immunoblotted with antibodies specific to the C-terminus of Hsp90. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

IV.9 References

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